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J. Martins e Silva
Carlota Saldanha

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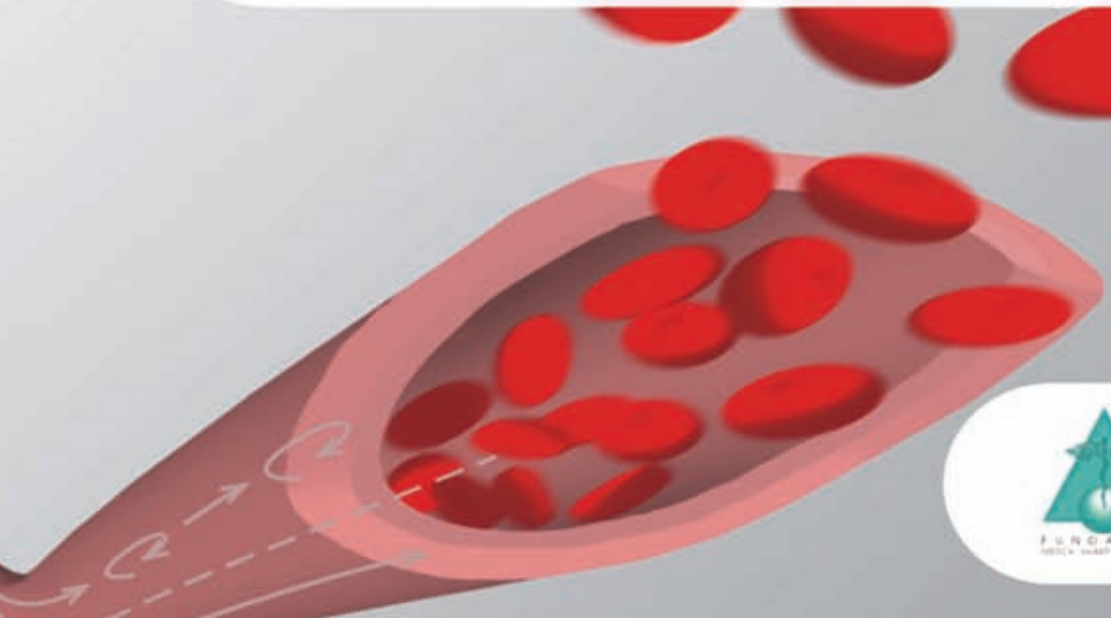

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INTRODUCTION TO ENDOTHELIAL CELLS MECHANOBIOLOGY

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SUMMARY

Almost all of the cells of the human body are subjected to mechanical stresses. In endothelial cells, mechanical stresses can vary from some milli-Pascal (shear stress) to some Pascal (hydrostatic pressure). Today it is know that mechanical stresses have a decisive part in cellular physiology. However, if the main biological effects of mechanical stress are well documentated, the mechanisms between mechanical forces to physiological phenomenon remain nearly unknown (mechanotransduction phenomenon). In this work, through personal results and published works, the authors considers the effects of mechanical stresses and possible hypothesis.

Key-words: endothelial cells; mechanobiology; mechanotransduction

1. BIOMECHANICS: OLD HISTORY AND DEFINITIONS

The interest of man for the explanation of the movements of the body has always been sharp. Famous names marked out the history of Biomechanics among them, without being exhaustive: Aristote (384-322 before JC) with the movement of the animals, Archimede (287-212 before JC) and Galien who were undoubtedly the first biomechanicians.

Since Léonard de Vinci (1452-1519) published his work on the human body movement and William Harvey (1578-1658) described the microcirculation and capillary circulation and can be considered as the first modern hemorheologists (He writed "*How does the blood manages to cross the porosities of the flesh one his way from arteries to the veins*?).

It has been then necessary to wait until the 17th and 18th centuries for a better comprehension of the physico-hemomechanics of blood circulation with Malpighi (1628-1694) who described the capillaries and red blood cells, Van Leeuvenhoeck who invented the first microscope or Malpighi and Hales (1677-1761) who measured blood pressure.

The first physiological, mechanical and hemorheological approaches date in fact from the 19th century. Jean-Marie Léonard Poiseuille (1799--1869) can be considered as one of the pioneers of modern physiomechanics. Her works allowing an approach of the complexity of the biological fluids and particularly of blood. The term of Biomechanics has been used for the first time in 1887 by Benedikt (Uber mathematische Morphologie und Biomechanick). In the same time works of Wolf on the adaptability of bone Roux on tissue remodeling, Fahraeus on the microcirculation, Burton, Copley, Scott-Blair, Born, Fung, Chien, Skalak helped to a better understanding of the role of the local forces on cell physiology and tissue remodelling.

Finally the progresses of the knowledges in Biology at the end of the 20th century by researches in Genetic and Molecular Biology, together with the development of physical instrumentations (atomic microscopy force, confocal microscopy, optical tweezers...) give a novel interest for the development of a new Biomechanics and its applications (cell and tissue Engineering, tissue and cell therapies...).

Biomechanics is the mechanic applied to life sciences. This definition is both vast and vague; it is also restrictive, because it does not include the physiological or pathological effects generated by the applied forces. Three definitions (more or less complete) can define the fields of biomechanics:

The former suggested by the application of the laws of mechanics aims to find solutions to solve medical, biological, ergonomic or sporting problems.

The second, more mechanical in its spirit, takes into account the study of mechanical properties of cells and tissues with regard to the complexity of the studied structures (ex: properties of the cardiac muscle, the wall of the vessels, the blood microcirculation, the cartilage, the bones...).

The later, more recent integrative, is interested not only in the properties of the objects studied through their structures, but also in their functions and their physiopathological consequences. This approach implements, not only the resolution of fundamental problems and development of models, but also the most recent knowledge of Molecular Biology, Genomics or Cell Biology. Its potential applications in cell and tissue engineering which, can be included under the term of "mechanobiology", seems very promising.

2. MECHANOBIOLOGY – MECHANOTRANSDUCTION

Cells in the body are permanently subjected to different forces (blood pressure, forces related to the movements...). Those vary from Pascal – Pa – (stress shear on the vascular wall) to MegaPa

– MPa – (forced on the cartilage of the hip). Recently however, it was shown that these forces were likely to influence the properties of cells (physiology, syntheses, expression of genes...) as well as biochemical modifications. One of the first observations, left a long time without continuation, was done by Wolff, a German surgeon who studied the adaptability of the bone. At the same time, Roux (1881) proposed the concept of adaptation which defined tissue remodelling.

At the interface between Physics and Biology, cell mechanics knew a conceptual revolution during the 20 last years with the possibility to measure and apply forces of picoNewtons and nanometric deformations. It was then possible to better understand the relation between local mechanical parameters and cell functions (concept of Mechanobiology).

If the biological effects of mechanical forces on cells and tissues are now relatively well described, the mechanisms explaining the passage from a mechanical stimulus to a physiological phenomenon (ex: secretions, receptor expression, gene activation...) remain difficult to understand. It is widely admitted today that these phenomena proceed in 4 steps.

a) *mechanical coupling* which induces transformation of the applied force into a detectable force by the cells or induction of a physical phenomenon (ex: pressure on a bone which induces a circulation of fluid in the canicular system or appearance of an electrokinetic potential of flow).
b) *mechanotransduction* itself which corresponds to the action of forces on specific structures. The transduction of mechanical signals could affect the properties of the cells.
c) *transduction of signal*: conversion of mechanical signals into intracellular physiological signals.
d) *cell response*: regulation of genes, release of autocrine or paracrine factors, expression of specific receptors...

If steps 3 and 4 are almost well described, knowledge of steps 1 and 2 requires development of models and experimental approaches specific to each type of cell studied (ex: distribution of forces on and into the cell, polymerization and orientation of the cytoskeleton...).

3. TRANSDUCTION INDUCED BY THE MECHANICAL FORCES ON VASCULAR ENDOTHELIAL CELLS (EC)

3.1. Mechanical forces acting on the vascular wall

The mechanisms by which endothelial cells identify the mechanical forces (shear stress or pressure) and convert them into physiological and biochemical signals remain generally unknown. Molecules at the cell surface are ideal candidates because they have a direct interaction with circulating blood. These molecules can be directly activated by a physical movement (conformational change) or indirectly by molecular gradients (which change ligand-receptor interactions). These membrane structures or “mechanoreceptor candidates” include integrins, ion channels and G proteins, tyrosine kinase receptors... These mechanoreceptors can trigger biochemical cascades in the cytoplasmic side of the plasma membrane through to second messengers release (centralized mode of the mechanotransduction). Thus activation of the proteins kinases is followed by stimulation of cytosolic transcription factors, and/or regulation of the gene transcription in the nucleus.

Another way to transduce the signal induced by stretch or shear stresses is related to the interaction of mechanoreceptors with cytoskeletal elements themselves activated by flow. By using a “decentralized” mode of mechanotransduction, transmission of signal can be done by connection with the cytoskeleton (focal adhesion, cell-cell junctions, membrane receptors) and leads to a

greater diversity of cell responses. Some of these responses induced by molecules of intracellular signalization (second messengers) are fast, of about a second or a minute (changes of the ion permeability, production of inositol triphosphate, intracellular release of Ca^{2+}, activation of the adenylate cyclase...), while other responses are developed in hours following the signal (genes modification of the cytoskeleton, changes of the cell shape and orientation...).

In vivo the vascular endothelial cell (EC) is subjected to 3 types of mechanical forces whose intensities are variable according to the vascular bed: shear stress (τ, some mPa), hydrostatic pressure (p, some Pa) and periodic parietal deformation (ε, 0 to 3 Hz).

The shear stress (τ) induced by the blood flow acts tangentially on the EC. It is generally determined by the relation of Poiseuille: $\tau = 2Q\,\mu / \pi D$, where Q is the flow rate μ the dynamic viscosity and D the diameter of the vessel.

The hydrostatic pressure acts perpendicular to the surface of the endothelium. It operates the extracellular matrix as well as on the endothelium. At the macroscopic scale, the blood flow in the arteries is generally laminar. However, the geometry of vessels near vascular singularities (junctions, stenosis...) predispose with separation of the flow and appearance of swirls. In these areas, the shear stress and the pressure present great fluctuations in amplitude and direction on short distances. At the microscopic scale, it should also be noted that the distributions of the shear stress and of the pressure on the surface of each EC are not uniform and are dependent on the topography of cell surface (Waché and Al, 2000).

In addition to the shear stress and the pressure, the arterial endothelium is submitted to a periodic deformation of the wall related to cardiac pulsations. The deformation (□) wide is classically measured is the average circumferential deformation, i.e. the relative variation of diameter: $\square = (D\text{-}D_0 / D_0$ with $D\text{-}D_0$ diameter between systole and diastole).

3.2. Effects of the shear stress on the EC: mechanotransduction

In 1968, Fry described modifications of the vascular endothelium in relation to the wall shear stress. In 1981, Dewey and al. showed the dynamic response of EC with regard to shear stress and Nerem and al. suggested that the morphology of the EC could be an indicator of the local hemodynamic conditions. During the last ten years, the influence of the local hemodynamic conditions on the EC, raised and increasing interest. The most recent works underlined the determining role of local flow conditions in their properties (Reinhart 1994, Davies and al. 1997 a), Ballermann and al. 1998). These modifications are described under the term of mechanotransduction. It is admitted that endothelium is the main actor in the processes of signal transduction that control vasomotion and various functions of vascular wall (Davies and al. 1997 b).

3.3. Distribution of mechanicals forces

The variations of the distribution of shear stresses can help to explain the various responses of endothelial cells to flows. Full-course of the endothelial cells being “rough”, the sensitivity of a cell can be considered by the fraction of surface exposed to a force above a critical value.

In addition to the variations of the distribution of forces between cells, the distribution of subcellular forces is variable in space. In this case, the localization of mechano-receptors or sensitive elements on cell surface can be a key factor. One can imagine that the sensitivity of a cell is determined by its form as well as by the localization and activation of mechano- elements on cell surface. Moreover, if we agree with the hypothesis of the presence of mecano-receptors on the membrane surface, their distributions are also a determining factor. A membrane receptor would be sensitive to the flow depending to the level of the forces in the areas. Thus, a particular flow would

not cause a cell response if its receptors are localized in a region where the force is lower than a defined threshold (Satcher and al. 1992, Barbee and al. 1995, Davies and al.. 1997-a and b).

3.4. Responses of EC to shear stress

a) Morphology of cells EC

The morphological changes corresponding to the lengthening and the orientation of EC parallel to the direction of the flow and the reorganization of the cytoskeleton are responsible for motility and cell adhesion (Dewey and al. 1981, Nerem and al. 1981, Ookawa and al.. 1993, Thoumine and al. 1995, Cucina and al. 1995). With low shear stress, in vivo and in vitro as well, the EC shows a polygonal form and are lengthened in comparison with the EC subjected to a high shear stress (Franke and al. 1984, Walpola and al. 1993, Drenckhahn and Ness 1997). In addition, the morphological changes of ECs are variable according to their localization (connections, junctions) and the conditions of flow (laminar periodic or disturbed flows, presence of vortex, etc.) (Davies and al. 1986, Helminger and al. 1991, Sato and Ohshima 1994, Truskey and al. 1995, Chiu and al. 1998).

All the elements of the cytoskeleton (actin filaments, microtubules, intermediate filaments...) undergo a reorganization during the application of shear stress, influenced by the amplitude and the period (Sato and Ohshima 1994, Girard and Nerem 1995, Galbraith and al. 1998).

At rest, EC show dense peripheral bands (DPB) with the stress fibbers ending in DPB areas. Proteins related to the cytoskeleton, such as the vinculins, Zyxins, VASP (vasodilatator stimulated phosphoprotein) etc. are associated at the ends of actin filaments to the periphery. Following a prolonged shearing, the EC are oriented in the direction of the flow. In cell, the stress fibbers and microtubules, as well as the intermediate beam alignment of filaments are aligned according to direction of the flow. This reorganization of the cytoskeleton will have a direct action on various cell signalling, in particular on protein phosphorilisation (such as the VASP) known to be the substrates of the proteins kinases A (PKA), or enzymes implicated into mechanotransduction. It will also have an effect on the properties of the endothelial cells, such as adhesion, release of Von Willebrand factor,...

b) Phosphorylation of the Vasodilator-stimulated Phosphoprotein (VASP)

The VASP are closely related to the reorganization of the cytoskeleton in the development and the repair of the endothelium, this phenomenon is important in the comprehension of vascular diseases. Phosphorilation of the VASP, triggered by a mechanical force, results in binding of VASP to the adhesion focal points of molecules of the cytoskeleton, such as vinculin or actin. Study of the phosphorilation of proteins in cells subjected to shear stresses of various durations and amplitudes as well as the analysis of the interactions between VASP and actin fibbers allowed us to demonstrate the existence of various phenomena and the presence of interactions between cytoskeleton and VASP.

c) Localization of adhesion molecules

We observed, also that EC activation, involves an increased expression of adhesion molecules such as ICAM-1, (ligant of leucocyte integrin) which support firm adhesion. In addition, during the application of a shear stress, ICAM-1 migrated towards the apical pole of the EC via the cytoskeleton to create an open area accessible to circulating adhesive cells (leucocytes...).

d) Regulation of Von Willebrand factor (VWF)

The von Willebrand factor is a multimeric glycoprotein (GP) wich is involved in of endothe-

lial lesion events, in platelet adhesion... It is also the transporter for the procoagulant factor VIII. It is synthesized in the Weibel-Palade bodies and is considered as a good marker of EC. It also plays a significant role in the atherosclerosis process which is itself in direct relationship to the mechanical forces of shearing on the wall. We showed an increase of vWF synthesis in cells exposed to a strong shear stress (1,0Pa) during 24h, but not in cells exposed to a low force (0,2Pa) showing that the local hemodynamic conditions (variation of the shear stress in vascular stenosis, etc) play a determining role in thrombosis and the atherogenesis via regulation of the release and the synthesis of vWF whereas the TNF-α induced a release of vWF simply.

e) Translocation of Caveolin-1

The caveolae are constituted by membrane microdomains implied in the mechanisms of mechanotransduction, due to their localization at the level of the ion channels and their properties of transport of macromolecules. Caveolin-1, constitutive protein of these microdomains, play a role as element of activation of the transduction molecules. Variations in their expressions were studied following to various mechanical and biological stimuli (TNF-α). We thus found a significant modification in the distribution and expression of caveolin-1 in EC exposed to a laminar flow and change in the spatial distribution according to time. More precisely, the caveolin-1 concentration was higher in the areas of high shear stress. Moreover, caveoline-1 expression increased following 24h shearing. On the other hand, TNF-α induced a reduction in the expression of caveolin-1 following 24h stimulation and inhibition of F-actin polymerisation blocked the redistribution of caveolin-1. These results show that the shear stress induces a translocation of caveolin-1 and that there is a correlation between this redistribution of caveolin-1 and the organization of F-actin in the EC.

It is possible that the molecules responsible for the transduction of mechanical signal are activated during their separation with caveolin-1. Consequently, changes of conformation or localization of caveolin-1 by shear stress could play a significant role in mechanotransduction.

f) Intracellular Responses

Since about years, the response of EC to mechanical forces was largely studied and the variations of a high number of cell functions were reported (electrophysiology, biochemistry, receptors, regulation of gene, etc) (Reinhart 1994, Davies and al. 1997-a). The effects of brutal or chronic shearing were studied *in vitro* (Ballermann and al. 1998, Braddock and al. 1998). The various responses of EC to mechanical forces can be classified according to the reaction time, although they are often simultaneous (Table I). For example, the fast electrophysiological changes in the membrane potential (of about a second) and the activation of the biochemical cascades occur in a similar characteristic time. G protein activation, release of NO, mobilization of derived the phosphoinositides, release of intracellular CA^{2+}, phosphorylation of cyclic nucleotides, etc, require longer times.

g) Gene regulation

Regulation of gene expression of molecules synthesized by the EC, like ET-1 (Morita and al. 1994), PAF (Diamond and col. 1990), PDGF A and B (Hsieh and al. 1991), MCP-1 (Shyy and al. 1994), adhesion molecules (ICAM-1, VCAM-1) (Nagel and al. 1994, Sampath and al. 1995, Ando and al. 1994), is influenced by the flow. Thus, expression of PDGF-B and FGF are increased in the EC and in the muscle cells smooth (CML) vascular when subjected to forces of shearing. Resnik and al. showed that the expression of PDGF-B in EC is dependent on a sequence of 12 nucleotides (SSRE) in the

Table 1 – Main responses of the EC to shear stresses

Time	Responses	Physiological significance	Authors
seconds	Activation of the channels potassic	– Selective opening of the channels K^+ – The entrée of calcium supports ??	Olesen and al.
	Activation of the seconds secondary messengers (IP_3, DAG, $CA^{2+,}$ PKC, protein-G...)	– activation of the transduction of signal (e.g. activation of the way Ca^{2+}- dependent)	Prasad and al. Bhagyalakshmi and al. Shem and al. Helmlinger and al. Hsieh and al. Berthiaume and Frangos Kuchan and al.
	Release of NO	– Vasorelaxtion flow – dependent	Kuchan and Frangos Gooch and Tennant
minutes	Release of PG	– Vasodilatation and anti-thrombosis	Frangos and al.
	Activation of MAP Kinase	– Transduction of the signal	Tseng and al.
	Activation of NFκ b Regulation of SSRE-dependent PDGF-B	– Activation of the transcription – Régulation cell multiplication	Mohan and al. Hsieh and al.
>1 h	SSRE-dependent Regulation (PDGF, OUR, tPA, TGFβ1, ICAM-1, c-fos, MCP-1 etc)	– Regulation of the growth cell – Vasorelaxation flow – dependent – Increase in the activity fibrinolytic – Cell adhesion – Transduction of thesignal – Recruitment of monocytes	Hsieh and al. Xiao and al. Diamond and al. Ohno and al. Nagel and al. Sampath and al. Hsieh and al. Shyy and al. Stoltz et al
>6 h	ET-1: increase (weak shearing); reduction (>0.6Pa)	– Vasoconstriction	Kuchan and Frangos
	VCAM-1: reduction	– Celle adhesion	Ando and al.
	Rearrangement of Cytoskeleton Alignment of the sites of focal adhesion Increase in the connexine 43 Rearrangement of organelles cell (MTOC, Golgi, etc).	– Mechanisme in the variations of morphology	Galbraith and al. Davies and al. Davies
	Cell prolifération Increase: turbulent disturbed No change or reduction: laminar	– Prolifération of CE	Davies and al. Gooch and Tennant
	Change of morphology (>12h) Rearrangement of Fn	– Adaptation to flow	Davies Barbee and al. Wechezak and al.
	Negative regulation of Tm	– Anti-thrombosis	Malek and al.
>24 h	Increase in the rigidity of cell surface	– Reduction in deformability cell surface	Sato and al.
	Alignment et elongation of the CE.	– Morphology adapted to flow	Girard and Nerem Galbraith and al.

promoter region of PDGF-B gene while is distinct from sequences of interaction with well know factors transcription. This same team showed that in another gene, coding for ICAM-1 which is involved in inflammation, increased when EC were subjected to forces of shearing: Whereas expression of VCAM-1 and ELAM-1, whose promoter region are deprived from SSRE sequence, was not affected under the same conditions. These observations suggest that the SSRE sequence was not affected under the same conditions. These observations suggest that the SSRE could be present in the EC and activated by mechanisms of mechano-transduction. The other transcription factors which take into the activation of promoters by shear stress are the nuclear factor kappa B (NF-KB), the activating protein-1 (AP-1), the early-1 growth promoter (Egr-1) c-fos, c-jun, c-myc and of stable (Sp-1) (Resnick and Gimbrone 1995, Gimbrone and al. 1997). The variations observed in gene regulation suggest that two types of gene elements sensitive to shear stresses (positive and negative) may exist end moreover, of the multiple regulations in company of the other factors transcriptional.

The response of gene expression of the EC to flow forces can be classified in tree types: early transitory increase, continuous increase expression of m RNA and biphasic regulation: Table II summarizes the different levels of regulation of molecule or gene transcription (level of ARNm) of molecules by the shear stress.

4. POTENTIAL MECHANORECEPTORS

The response of EC to mechanical stimuli relates to practically all the mechanisms dependent on growth, cell metabolism and their functionality. However, the mechanisms remain hypothetical: how and by which “sensors” on the EC re-

Table II – Regulation of the transcription (level of ARNm) of genes by a shear stress (according to Braddock and al. 1998 and Stoltz and al. 1999).

Genes	Cells used	Response of the ARN	SSRE	Other factors
ET-1	HUVEC/BAEC	Biphasic	–	AP-1
VCAM-1	HUVEC	decrease (τ strong) increase (τ low)	–	AP-1, NF-κB
ACE	RAEC	decrease	+	SSRE,AP-1,Egr-1
TF	BAEC	increase	–	Sp-1
TF	HAEC/HUVEC	increase	–	Egr-1
Tm	HUVEC	Biphasic	–	AP-1
PDGF-A	BAEC	Biphasic	+	Egr-1
PDGF-B	BAEC	Biphasic		
ICAM-1	HUVEC	increase (or biphasique)	+	AP-1,NF-κB
TGF-β	BAEC	increase	+	AP-1,NF-κB
c-fos, c-jun	HUVEC	increase precociously transitorily	+	AP-1
ENOS	HUVEC	increase	+	AP-1,NF-κB
MCP-1	HUVEC	Biphasic	+	AP-1,NF-κB

ACE – Angiotensin-converting enzyme; TF – tissue Factor; c-fos and c-jun – members of proto-oncogen family; eNOS – endothelial Nitric Oxide Synthase; AP-1 – Activator Protein-1; NF-κB – Nuclear Factor-κB; Egr-1 – Early Growth Response Factor-1; SSRE – Shear Stress Response Element.

ceive these mechanical stimuli and convert them into biochemical signals?

The molecules present at the luminal cell surface are at first sight the ideal candidates because they are in direct contact with circulating blood. These molecules can be activated directly by a physical displacement (conformational change) or indirectly by transfer of gradients (which change the interactions ligant-receptor). These membrane structures or mechanoreceptors include ion channels, integrins, G proteins related to the receptors and receptors tyrosine kinase, caveola...

These mechanoreceptors can induce cascades of response from the plasma membrane, through release of biochemical second messengers (centralized mode of mechano-transduction). Thus activation of the proteins kinases is followed by stimulation of factors of cytosolic transcription, and/or regulation of gene transcription in the nucleus (Patrick and McIntire 1995).

Another way of signal transduction can be related to the interaction of mechanoreceptors, activated by flow, with elements of the cytoskeleton. By using a "decentralized" mode of mechanotransduction, the transmission of the signal would be induced then via connection with the cytoskeleton (sites of focal adhesion, junctions cell-cell, nuclear membrane) which would lead to the great diversity of cell responses.

Some of these responses are fast, of about a second or of a minute. Other responses develop in the hours following the birth of the signal (cf Table I).

4.1. Integrins

The integrins are responsible for cell adhesion and migration on the extracellular matrix. Via their interactions with other molecules, they initiate the modulation of cytoskeleton organization. They are largely implied in the regulation of the embryonic development, apoptosis, hemostasis, recruitment and activation of the leucocytes and retraction of the blood clot. At the time of connection to integrins, the ligands bind firmly or gather the integrins while binding to the adjacent molecules on the cell surface. The connection or the assembly of integrins leads to the formation of focal adhesion where the integrins bind to intracellular cytoskeletal complexes and indirectly with the actin filaments. The displacement of a transmembrane integrin could communicate the force with the cytoskeleton through protein/protein interactions in the cytoplasmic side of membrane. For example β1 integrin receptor of extracellular matrices can induce the formation of a focal adhesion and induce a force-dependent signal. The rigidity of the cytoskeleton would increase with the applied pressure, thus would require intact microtubules as well as intermediate filaments and microfilaments.

Following this connection closes integrins with their ligands, they "integrate" the signals external given by the othercells or the components of the extracellular matrix to which they adherent, signals which they transmit inside the cell while joining the cytoskeleton and by starting signals of transduction. These signals lead to the hydrolysis of phospho-inositols and thus to the increase in intracellular Ca^{2+}, with phosphorylation of several proteins, in particular activation of tyrosine kinase of focal adhesion ($pp125^{FAK}$ and to the induction of various genes.

The application of a shear stress causes the fast activation of protein kinases, among which ERK (extracellular signal-regulated kinase) and JNK (c-Jun-terminal kinase) (Li and al. 1996). Whide lead to the transcriptional activation of early genes such as those coding for MCP-1 (monocyte chemotactic protein-1) and c-fos (Shyy and al. 1994, Jalali and al. 1998). These activations are modulated by the Ras protein, whide is itself controlled by S.O.S. protein (its of sevenless). However, in response to many growth factors such as the PDGF (platelet derived growth Factor) or the EGF

(Epidermal growth Factof), the protein adaptor Shc (Src homology2/alpha collagen) is phosphorylated at the level of its tyrosines and interact with phosphor-tyrosines of receptor Tyrosine Kinase through binding to SH2 (Src homology domain-2). Following phosphorylation, it can also interact with G2b2 (Growth Factor receptor--binding protein-2) through SH2 binding. Shc--Grb2-S.O.S. provides then an alternative way of signalization in addition to Grb2-S.O.S. transduction used by the Ras protein.

Recently it has also been shown that the Shc protein is implied in the transduction signals.

4.2. Ion channels

The lipid bilayer of cell membranes has a great permeability to polar molecules of small sizes and hydrophobic molecules while it is highly impermeable to ions and charged molecules. Specialized membrane proteins (channels and transporters) are responsible for the specific transfer of ions through the membrane. The ion channels and the ion exchangers are thus potential mechanoreceptors (Davies 1995). The ion channels K^+ modified by the stretching of the membrane would have their activity modified in response to a mechanical force. Thus, Olesen and al. identified a current K^+ selective by activated by the shear stress. This polarizing membrane current is a function of the shear stress, reaching half of its maximum of activation with 0,7 mPa.s. It is quickly activated by the shear stress (a few seconds), slowly increases (in a few minutes), and completely returns to the normal when the flow is stopped. Nevertheless, it is not certain that the activation of these channels is a primary response to the shear stress.

By the same way, many channels are used by calcium which can activate many ways of signalization, among which one particularly stimulates the production of NO and consequently vasodilatation of vessels (Himmel and al. 1993). It was proposed that the mechanisms depending on intracellular calcium concentration can play a significant role in the early and transitory responses, whereas the mechanisms independent of $[Ca^{2+}]I$ changes would be significant in the late and prolonged responses.

The calcium-dependent way is likely to be responsible for the fast response and the transitory flow, such as fast activation of NOS. In addition, intracellular calcium plays a crucial role in the reoganization of the cytoskeleton and the alignment of the EC subject to a flow (Malek and al. 1996). On the other hand, the calcium--independent way induces activation of GTPases binding to GTP and stimulation of PKC and calcium-independent MAP kinase.

4.3. Receptors link with the G proteins

Stimulation of many membrane receptors is retransmitted by a class of specific proteins, that blind to GTP (Guanosine Tri-Phosphate), those are G proteins. They operate the coupling of the receptors with the intracellular effector, and for this reason exert a significant control on the transmission of the signal. The interactions between the receptors and their second messengers are mediated by enzymes or ion channels activated by the interaction with G proteins. G proteins are heterotrimeric proteins, constituted by a sub-unit α bound with the heterodimer βγ. The α sub-unit binds to GTP, hydrolysis in GDP (Guanosine Di-Phosphate) then induce the response of the majority of the effectors. A receptor coupled to a G protein at rest is activated by the binding of a specific agonist. The change in the conformation of the agonist--receptor complex, induced by this interaction, allows the activation of the exchange of GDP by GTP and thus activation of the G α and G β/γ sub--units which will control the membrane or cytosolic activity of various effectors. The release of the phosphatase activity, wihin the G α sub-unit induces the reassociation of the G α and G β/γ sub-units and leads to a return to the initial state.

The amplification of the signal triggered by an extracellular modulator follows two steps. The former is related to the activated receptor which can activates many G proteins in cascade; in the later, the α-GTP sub-unit maintains the amplified activation as long as the GTP is not hydrolyzed in GDP. Recently, it was been shown (Gudi et al. 1996) that activation of G protein is one of the earliest events in the signalization induced by flow. Following this work, a new study highlighted selective changes specific to the nature of the G protein stimulated, in correlated with changes in the signalization and the functionality of G protein (Redmond and al. 1998). Thus, receptors related to the G proteins can be also considered as potent-transducers and, downstream, as signal devices.

4.4. Receptors of tyrosine-kinase (RTK)

In fact transmembrane proteins, following stimulation, induce signalisation events. Their common characteristics is the presence of a single transmembrane segment and an intracellular field having catalytic activity of the protein tyrosine--kinase type. The interaction of ligands involves the dimerization of receptors which allows the activation of kinase carried by each of the two chains and the assembly to the adjacent sequence target also carried by the two chains. This allows intermolecular cross phosphorylation at several tyrosine residues. The phosphorylated dimmer represents the activity receptor. It contains a whole series of phosphorylated tyrosine residues which have the capacity to bind to proteins and form signalization complexes. Moreover, the dimerized and phosphorylated receptor has the potential to phosphorylate its targets. The analysis of the sequences which bind to the phosphorylated receptors showed that the majority of them, but not all, contain SH2 domains. Of other contain PTB (PhosphoTyrosinee Binding Protein) domains. Thus, assembly of the complexes of signalization depends on recruitment by phosphorylated tyrosines of the protein receptors having of a fields SH2 or PTB. Among many proteins containing of SH2 domains binding the RTK domains to form complexes. Some of these proteins themselves are phosphorylated at the end of this association.

4.5. "Mechano-sensors" and Interactions

In order to identify new interactions between various mechano-sensors, we particularly analyzed two different steps of the of mechano--transduction phenomenon: the early response, the interactions integrins-shc and a step downstream, relative to the JNK activity (Labrador and al., 2002)

With the help of specific inhibitors of the site of integrins interactions (RGD), intracellular calcium (BAPTA/AM) and G_i proteins (PTX), we confirmed the existence of interactions within the EC to allow the adaptation of the cell to mechanical forces. Indeed, the exposure of the EC to a laminar flow constant armature resulted in the fast formation (in half an hour) of new intracellular complexes between the integrins αv β3 and the protein Shc. This response uses the same process as that of the biochemical signal i.e. through interactions of integrins to their ligand. Moreover, the information of these complexes in response to flow is partly dependent on the intracellular concentration of calcium but also on the activity of G_i proteins. This step is in addition essential to the later response of the EC to shear stresses such as activation of JNK, via the integrins interaction, the intracellular presence of calcium and the catalytic activity of the G_i proteins.

Mechanical forces would activate at a first step mechano-sensitive sensors. This initial steo would be followed by with the intervention of second messengers: Intracellular Ca^{2+}, protein kinase C (PKC), G protein, Adenosine monophosphate (AMP) cyclic and guanosine monophosphate

(GMP) cyclic, etc. These changes in the balance of the second messengers then involve a change of the state of activation of "DNA binding factors" (Hsieh and al. 1992, Morita and al. 1994, Kuchan and Frangos 1994).

The final step results then in a damaging of the activity of transcription of many genes via brief replies to local shear stresses such as Shear Stress Response Element (SSRE) with positive or negative effects, or a combination of both, acting with various steps.

5. CONCLUSION

It is known today that mechanical forces induce many key events in the physiopathology of the vascular endothelium. To illustrate this phenomenon, Papadaki and Eskin proposed, in 1997, a first diagram summarizing the ways of signalization to "multiple responses" activated by shear stresses within the EC. The activation of one or several mechanoreceptors induced biochemical events which lead intracellular changes in the metabolic and gene expression of the cell, controlling thus the function. This concept, comparatively to the identification of mechanoreceptors and the responses of other cells (chondrocyte, osteoblasts) to mechanical forces should make it possible to a best understanding of mechanoregulation of cells.

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THE ROLE OF DLL4 IN THE REGULATION OF ARTERIOGENESIS AND ANGIOGENESIS

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ABSTRACT

Vascular development is dependent on various growth factors and certain modifiers critical for providing arterial or venous identity, interaction with the surrounding stroma and tissues, hierarchical network formation, and recruitment of pericytes. Notch receptors and ligands (Jagged and Delta-like) play a critical role in this process in addition to VEGF. Dll4 appears to be the most important of the Notch ligands in the regulation of arterial specification and vessel maturation events, as well as modulating the angiogenic response by controlling the abundance of tip cells. In this review we present various evidence supporting these claims and suggest that they indicate Dll4 as a promising target for therapeutic intervention in adult angiogenesis.

INTRODUCTION

Primary vasculogenesis serves as the template from which a higher order of branching network is generated by the process defined as angiogenesis.[1-3] During angiogenesis, branching of arterial and venous components is orchestrated such that the capillaries from these two compartments fuse in symmetry, anchored in place by interaction with matrix proteins.[4] Vascular endothelial growth factor (VEGF) is indispensable for the formation of the primary vascular network and for secondary angiogenesis.[5] However, it requires the presence of precise quantities of several other constituents within well-defined temporal and spatial constraints to construct and remodel the vascular system. Specifically the Notch signaling pathway is necessary to provide signals for phenotypic determination of arteries and veins, and regulated vessel migration and branching leading to the vascular morphogenesis and remodeling.[6]

In mammals, the Notch family of proteins is composed of four single-pass transmembrane receptors (Notch1-4) and five membrane bound ligands (Jagged1, 2 and Dll1, 3, and 4). Mutations of Notch receptors and ligands in mice and humans lead to abnormalities in the vascular system.[7] The Notch pathway functions through cell-cell interaction such that the extracellular domain of cell membrane–bound ligand interacts with the extracellular domain of the receptor on an adjacent cell. Notch receptor activation requires cleavage of Notch intracellular domain (NICD) and translocation to the nucleus, and activation of target genes.[8]

Differentiation of vascular cells to arterial or venous compartments was previously thought to depend on physical factors such as blood pressure and oxygen concentration. Over the past few years, however, the differential and restricted expression of a number of genes in arterial or venous endothelial cells prior to the onset of circulation suggested the potential for genetic determination of the arterial and venous fate of primary endothelial cells. Among these genes are *Notch1*,[9] *Notch4*,[10] *Dll4*,[11] and the Dll4-Notch-regulated genes *EphB4* and *EfnB2* specifically expressed in venous[12] and arterial endothelial cells, respectively.[13,14]

Vascular expression of Dll4 and its cognate receptors, Notch1 and Notch4, is restricted to arterial endothelium. *Dll4* is one of the earliest genes expressed in arterial endothelial cells, is induced by VEGF-VEGFR signaling, and is essential for the establishment of the arterial endothelial cell fate.[14-16]

DLL4 HAPLOINSUFFICIENCY

We, along with two other laboratories, have recently shown that the Dll4 ligand alone is required in a dosage-sensitive manner for normal arterial patterning in development.[16,17,18]

The *Dll4* gene was inactivated by targeted disruption in embryonic stem (ES) cells with a targeting vector designed to replace the initiation codon and the first three coding exons with the β-galactosidase (*lacZ*) reporter gene.[16] When 100% germline transmitting male chimeras were crossed with wild-type ICR females only 21% of the agouti offspring was $Dll4^{+/-}$, rather than the expected 50% frequency, indicating the death *in utero* of a proportion of the $Dll4^{+/-}$ F1 embryos. Outcrossing these viable heterozygotes to outbred ICR mice still resulted in a reduced number of heterozygotes in subsequent generations. This effect was dependent on the genetic background, as no live $Dll4^{+/-}$ offspring were obtained when the 100% germ-line transmitter chimeric males were crossed with inbred 129/Sv-CP females.

To further characterize this incompletely penetrant haploinsufficiency, we investigated the phenotypes of $Dll4^{+/-}$ embryos. In pre-somite stages, *lacZ* expression was exclusively detected in trophoblast giant cells. Around the two-somite stage (E8.0), the *lacZ* reporter was expressed in the cardiac crescent and the primordia of the dorsal aortae (Fig. 1A). At the five- to 10-somite stage (E8.5), it was expressed in the heart, paired dorsal aortae, branchial arch arteries, internal carotid arteries, umbilical artery, vitelline artery, and in the posterior region of the yolk sac where the arteries are formed (Fig. 1B,C).

While at E8.5 there were no obvious differences between wild-type and $Dll4^{+/-}$ embryos, as development proceeded, however, some heterozygous embryos started to show developmental delay. At E9.5 only 31% of the $Dll4^{+/-}$ embryos were normal in body size and somite number as the rest showed growth retardation. At E10.5, two types of $Dll4^{+/-}$ conceptuses were found: normal-sized embryos with reduced vitelline circulation, and severely retarded embryos with reduced vitelline circulation and enlarged pericardial space, indicative of embryonic circulatory defects (Fig. 1D–F). The caliber of the major vitelline arteries and the arterial branching on the yolk sac was reduced in all of the heterozygous embryos (Fig. 1I, J). The umbilical artery and placental blood

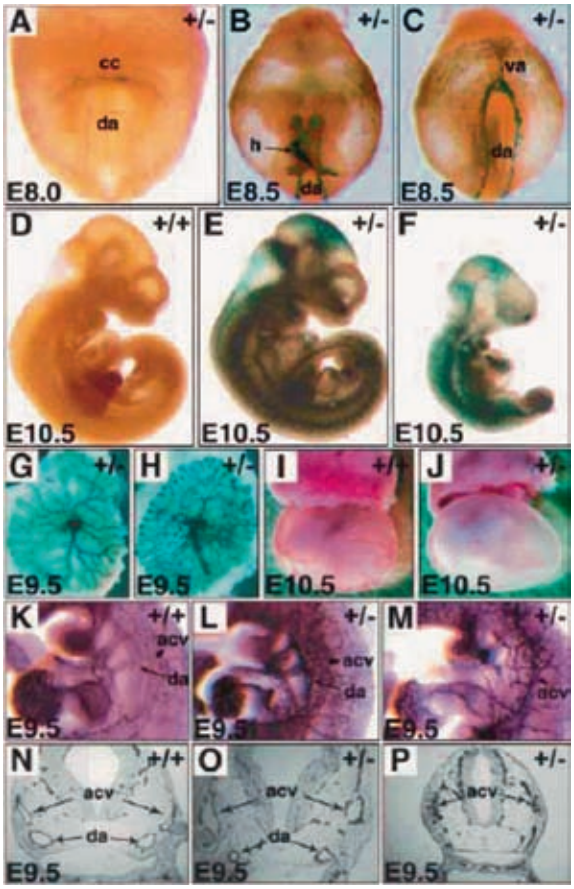

Fig. 1 – *Dll4* haploinsufficiency. (*A–H*) Whole-mount X-gal staining. (*K–P*) PECAM immunostaining: whole-mount (*K–M*) and sections (*N–P*). (*I, J*) Unstained specimens. (*A*) *Dll4* expression at two somite stage in the cardiac crescent (cc) and dorsal aortae (da) primordia. (*B,C*) At E8.5, *Dll4* expression is observed in all arterial vessels (da, dorsal aortae; va, vitelline arteries) and in the endothelial lining of the heart (h). (*D–H*) Variable penetrance of the hemizygous phenotype: littermate embryos (E10.5, *D–F*) and arterial vessels of the placenta (E9.5, *G, H*). (*I, J*) Morphology of E10.5 yolk sacs. Note the absence of large vitelline vessels and the poor circulation in the *Dll4*$^{+/-}$ yolk sac compared with wild-type. (*K–P*) At E9.5 two types of *Dll4*$^{+/-}$ embryos can be found: with reduced dorsal aorta but without defects in the anterior cardinal vein (*L,O*) and with more severe defects, with a very reduced dorsal aorta, enlarged heart, and the anterior cardinal vein reduced to a disorganized venous plexus (*M,P*).

vessels were also reduced in size in some *Dll4*$^{+/-}$ embryos (Fig. 2G,H). The variable phenotype of the placental vascular bed probably explains why some *Dll4*$^{+/-}$ embryos could survive to term, despite the poor vitelline circulation in all heterozygotes, since the dependence of the embryo on the vitelline circulation for gas and nutrient exchange is only transient until the placental circulation is established.

Defects in vascular structures were also seen within the *Dll4*$^{+/-}$ embryos, most notably the reduction of the caliber of the dorsal aortae. At E9.0 the dorsal aortae were already thinner than normal in the majority of the embryos, a feature which was accentuated at E9.5 and E10.5. The constriction of the dorsal aortae was primarily observed rostrally between the cardiac level and the intersections with the branchial arch arteries (Fig. 1K–P), but some embryos exhibited longer zones of constriction extending caudally (data not shown). At both E9.5 and E10.5, 90% of the embryos displayed some degree of aortic constriction.

The potential impact of these arterial defects on the development of the venous system was also investigated. Reduced caliber and disorganization of the anterior and posterior cardinal veins were observed in the *Dll4*$^{+/-}$ embryos, but only in those cases where the dorsal aortae were severely affected, suggesting that the venous defect is a secondary response to a primary arterial restriction (Fig. 1, cf. L,O and M,P).

Surviving *Dll4*$^{+/-}$ mice were apparently normal, suggesting that in these animals the major defects caused by reduction of Dll4 are transient. This allowed the intercrossing of *Dll4*$^{+/-}$ mice and subsequent examination of the homozygous null phenotype.

DLL4 LOSS-OF-FUNCTION

As expected, the *Dll4*$^{-/-}$ embryos showed more severe and precocious vascular defects than heterozygotes.[16] Mutant embryos were morphologically normal until E8.5. The correct migration and aggregation of the angioblasts occurred to form the dorsal aortae, showing no disruption of the onset of vasculogenesis. However, the dorsal aortae already showed a clear reduction in diam-

eter by E8.75 (Fig. 2A, B). By E9.0 the homozygous null embryos were highly delayed and abnormal, with severe pericardial swelling, and drastically reduced dorsal aortic diameter in the anterior region (Fig. 3E–H). Not only were the major arteries abnormal; branching morphogenesis was also affected. *Dll4*$^{-/-}$ embryos showed an abnormal accumulation of *lacZ*+ endothelial cells in the apical portion of the intersomitic vessels and an abnormally dilated dorsal vessel in this region (Fig. 2, cf. G and H). This defect would result in abnormal blood circulation in the embryo, with misdirection from the dorsal aorta to the lateral vessels.

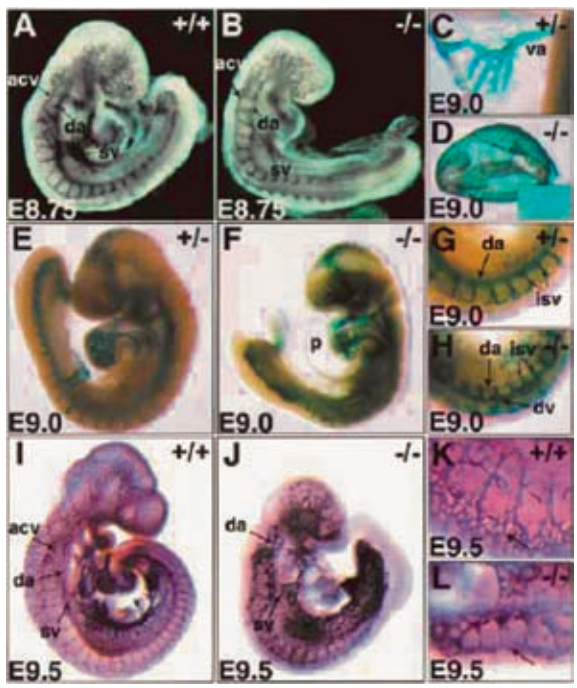

Fig. 2 – Defective arterial and venous remodeling in *Dll4*$^{-/-}$ embryos. Whole-mount PECAM (*A,B,I–L*) and X-gal staining (*C–H*) of control and *Dll4*$^{-/-}$ embryos. At E8.75, *Dll4*$^{-/-}$ embryos display reduced dorsal aorta (da), anterior cardinal vein (acv), and sinus venosus (sv). (*C,D*) Presence of vitelline arteries (va) in the yolk sac of a E9.0 *Dll4*$^{+/-}$ embryo contrasts with *Dll4*$^{-/-}$ yolk sac vessels, which appear stalled at the primary capillary plexus stage (*inset*); in *Dll4*$^{-/-}$ embryos the *Dll4-lacZ*reporter expression is observed throughout the yolk sac vessels. (*E–H*) By E9.0, *Dll4*$^{-/-}$ embryos exhibit growth retardation and arrested heart development with pericardial edema (p); the dorsal aorta appears further reduced, and there is an abnormal accumulation of *lacZ*-positive cells in the apical portion of the intersomitic vessels (isv) forming an enlarged dorsal vessel (dv). By E9.5, the *Dll4*$^{-/-}$ embryos are severely retarded with extremely reduced dorsal aortae, a reduced and almost indistinguishable venous structure, sinus venosus, and anterior cardinal vein. (*K,L*; higher magnification of *I, J*, respectively). In the more dorsal region, instead of an intricately branched network between arterial and venous intersomitic vessels, the *Dll4*$^{-/-}$ embryos display a single fused vessel that did not undergo angiogenic remodeling.

Yolk sac circulation was also highly abnormal. The vitelline artery was drastically reduced, and the yolk sac vascular plexus showed an angiogenic remodeling defect with persistence of the primary capillary bed. Interestingly, whereas in the heterozygous embryos the *lacZ* reporter was strictly expressed in the arterial (posterior) region of the yolk sac vasculature, in the null embryos, *lacZ* was expressed in all endothelial cells of the yolk sac plexus (Fig. 2C,D). Although vitelline arteries fail to form, *Dll4* expression is clearly activated in the yolk sac precursors, consistent with it being upstream of the genes that specify artery fate.

By E9.5 these phenotypic traits were accentuated, with more pronounced growth retardation and arterial atrophy, the dorsal aortae being absent or reduced to a rudimentary capillary plexus (Fig. 2I,J). The hearts in these embryos showed reduced atrial and ventricular chambers, and the ventricular trabeculation was markedly reduced.

The head vasculature consisted of a simple plexus of disorganized and fused vessels (Fig.2J). Venous development was also impaired in the null embryos. At E8.75 the anterior cardinal vein already appeared to have a reduced caliber and ectopic branching at some points, and the sinus venosus appeared smaller (Fig. 2A,B). By E9.0 the anterior cardinal vein was further reduced, and by E9.5, a distinct anterior cardinal vein was absent and the embryos showed a very reduced sinus venosus (Fig. 3I,J). Therefore, although the major arteries and veins of the embryo form in the absence of *Dll4*, their later development is severely disrupted. As *Dll4* expression is artery-specific, the venous defects are likely secondary to arterial patterning and growth problems.

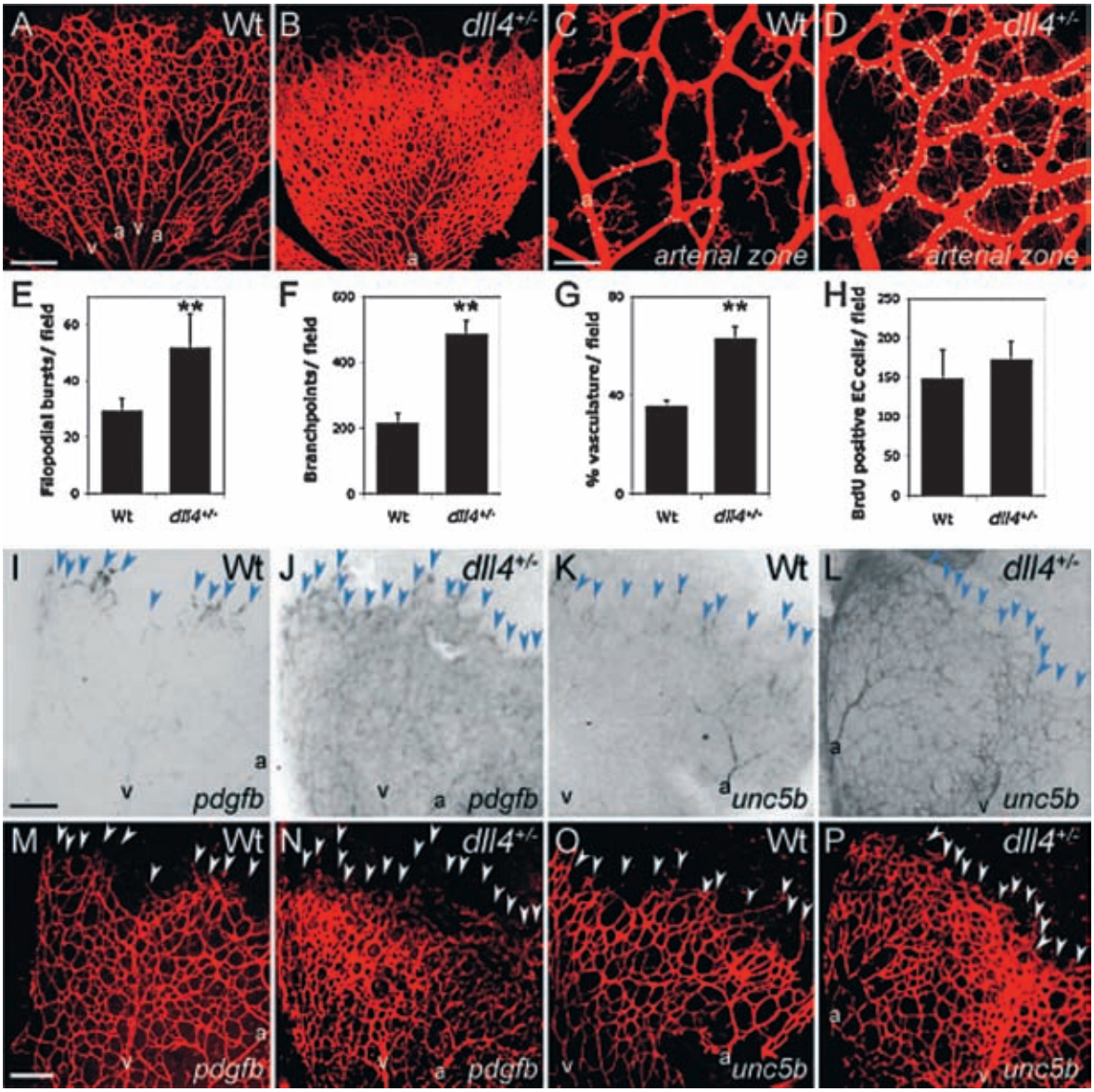

Fig. 3 – Increased tip cell formation in *Dll4*$^{+/-}$ retinal vessels. (*A* and *B*) Isolectin B4-stained P5 *Dll4*$^{+/-}$ retinal vessels show a hyperfused plexus compared with wild-type (a, artery; v, vein). (*C* and *D*) *Dll4*$^{+/-}$ vessels extend many more filopodia within the vascular plexus compared with wild-type (arterial zone shown; dots indicate filopodia extensions). (*E–H*) Quantification of filopodial bursts at the vascular front, branchpoints in the vascular plexus, percentage of retina covered by vessels, and BrdU-labeled retinal ECs in wild-type and *Dll4*$^{+/-}$. (*I–P*) Whole-mount ISH shows expanded expression of tip cell marker genes *Pdgfb* and *Unc5b* in *Dll4*$^{+/-}$ compared with wild-type (arrowheads indicate tip cell expression at vascular front). (*M–P*) Corresponding isolectin B4 staining of retinal vessels. Error bars, SD; ******, $P < 0.001$, Mann–Whitney *U* test. [Scale bars, 250 μm (*A* and *B*); 40 μm (*C* and *D*); and 100 μm (*I–P*).]

In zebrafish, Notch signaling has been implicated in the specification of arterial endothelial cells by suppressing the venous cell fate.[14] To investigate whether the disruption of vascular development in *Dll4* mutants could be at least partially attributable to abnormal identity of the vascular endothelial cells, we carried out RNA *in situ* hybridization and immunostaining to determine the expression of arterial and venous markers. In *Dll4*$^{-/-}$ embryos with residual intact dorsal aortae,

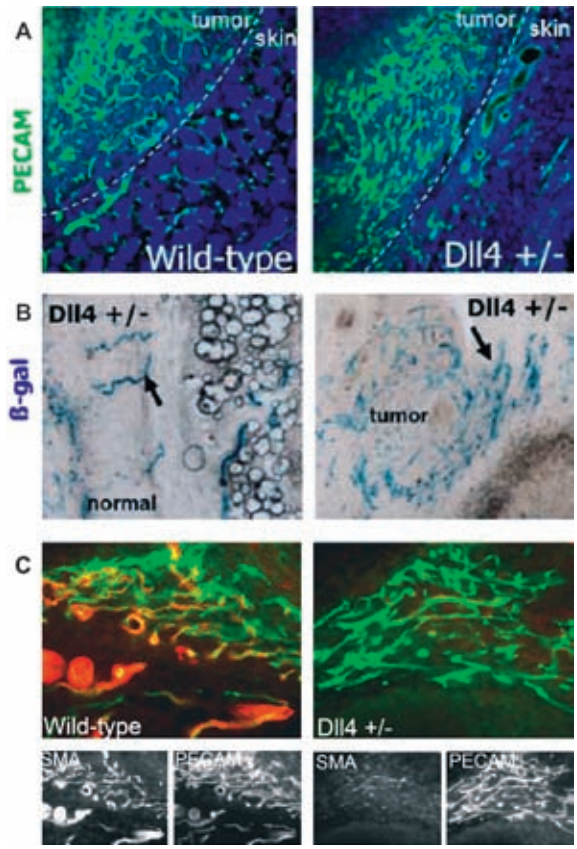

Fig. 4 – Increased tumor branching angiogenesis in *Dll4*$^{+/-}$ mutant mice: (A) Vascular response in ***Dll4***$^{+/-}$ adult mice was examined after tumor implantation. Wild-type mice showed organized vascular proliferation in the tumor (left half), while mutant mice showed markedly increased vascular response. **(B)** Expression of Dll4 in tumor and normal regions in Dll4+/- mutant mice was examined by ß-gal staining. Dll4 expression was observed in a few discrete vessels in the normal tissue, while the tumor region showed many ß-gal positive vessels of similar appearance, indicative of *Dll4* induction in tumor vessels. **(C)** Pericyte coverage around newly forming vessels was examined by α-SMA localization. In wild type mice, the vessels showed co-localization of PECAM and α-SMA (left panel). In *Dll4*$^{+/-}$ mice tumor vessels however, the number of α-SMA positive cells lining the endothelial cells was profoundly reduced (right panel).

the VEGF receptor, Flk1, was normally expressed in both arteries and veins, and the *Dll4-lacZ* reporter was expressed in the arteries. However, none of the downstream arterial markers studied (EphrinB2, Connexin37, and Connexin40) were expressed in the endothelium (data not shown).

This is consistent with the proposed pathway for zebrafish, where VEGF signaling upstream of Notch signaling promotes arterial cell fate.[14] In addition to loss of arterial markers, the venous marker EphB4 was ectopically expressed in the dorsal aortae as well as in the cardinal veins (data not shown). In some null embryos, from E9.0, the dorsal aorta fused with the anterior cardinal vein at the level of the sinus venosus (data not shown), consistent with possible loss of separate identity of the two vessels. These data strongly suggest an involvement of the Notch signaling pathway, mediated through the Dll4 ligand in a cell-autonomous manner, in the establishment of the endothelial arterial cell phenotype in mice.

The similarity of the *Dll4*$^{-/-}$ phenotype to that of the *Notch1/4* double mutants[9] is consistent with this, as is the contrasting effect of endothelial specific activated Notch expression.[19] Loss of arterial vascular identity in *Dll4*$^{-/-}$ mutants, in turn, could cause angiogenic defects leading to a generalized disruption of the vasculature and embryonic death.

The strain-dependent haploinsufficiency of *Dll4* was surprising because no other component of the Notch pathway has shown such an effect on vascular development. Indeed, this is the first reported lethal haploinsufficiency for any Notch signalling component in mammals. However, vascular development-related haploinsufficiency has also been reported for the VEGF gene[5] which appear to lie upstream of Notch signaling in arterial development.[14] This suggests that the development and patterning of the arterial system may be controlled by levels of availability of critical ligands. Interestingly, both VEGF and Dll4 have been shown to be up-regulated by hypoxia,[20, 21] which is one of the environmental factors that can impinge on vascular patterning and growth. Presumably, exquisite sensitivity to ligand levels helps to ensure appropriate vascular responses to changing external environments. Sensitivity of the embryonic vasculature to Dll4 levels raises the possibility that Dll4 might be a good target for intervention in adult neovascularization.

DLL4 IN THE REGULATION OF ANGIOGENESIS

All *Dll4*$^{-/-}$ embryos examined showed branching defects but with varying severity, indicating that unknown genetic modifiers also influence the *Dll4*$^{-/-}$ phenotype. To exclude the possibility that the defects observed in vessel branching were secondary to other defects in the embryo, *e.g.*, disrupted blood flow, we cultured aortic explants from *Dll4*$^{-/-}$ and wild-type mice in collagen gels.[22] Sprouting from *Dll4*$^{-/-}$ aortas occurred earlier and more profusely than from wild-type aortas (data not shown), indicating that the *Dll4*$^{-/-}$ vessel defect is intrinsic to endothelial cells (ECs) and independent of alterations to flow or cardiac output.

To establish the underlying cause of the *Dll4*$^{-/-}$ vessel branching defects, we performed high-resolution confocal microscopy on isolectin B4-stained *Dll4*$^{-/-}$ and wild-type E11.5 hindbrains.[22] Staining for β-gal in hindbrains showed expression in virtually all ECs (data not shown). Vessel branching was significantly increased in *Dll4*$^{-/-}$ compared with wild-type hindbrains (data not shown). Strikingly, vessels from *Dll4*$^{-/-}$ hindbrains extended numerous filopodia from the whole length of the vessel surface, whereas vessels from wild-type hindbrains extended only a few filopodia at scattered points.

Filopodia extension is a morphological characteristic of specialized ECs called ''tip cells,'' which are lumenless ECs present at the leading edge of vascular sprouts that integrate directional cues from their environment and so define the direction in which the new sprout grows.[23] Tip cells also find and create connections with adjacent sprouts and so generate functional vascular networks.[24] The profusion of filopodia in *Dll4*$^{+/-}$ hindbrains suggested that most, if not all, ECs in the hindbrain vessels were acting like tip cells, leading to increased connections between adjacent vessels (*i.e.*, branching).

To further evaluate whether loss of *Dll4* leads to increased endothelial tip cell formation, we analyzed postnatal retinal vascular development of surviving *Dll4*$^{+/-}$ and wild-type pups.[22] The retina vasculature at postnatal day (P) 4–6 allows for simultaneous visualization of angiogenic sprouting at the vascular front (where most endothelial tip cells are located) and remodeling of the nascent vasculature within the vascular plexus.[25] In wild-type retinas, *Dll4* expression was detected predominantly in arteries and in tip cells at the vascular front, with lower levels observed in vessels within the vascular plexus (data not shown). In *Dll4*$^{+/-}$ vessels, lower levels of *Dll4* were detectable by *in situ* hybridization and quantitative PCR (qPCR; 51% of wild-type levels), although the expression pattern was comparable to wild-type.

As with hindbrains, *Dll4*$^{+/-}$ retinal vessels showed severe patterning defects, forming a hyperbranched, hyperfused plexus behind the vascular front (Fig. 3 *A* and *B*). Numerous filopodia extended from *Dll4*$^{+/-}$ vessels at the vascular front and also within the vascular plexus in both arterial and venous zones (Fig. 3*D*). In contrast, wild-type vessels extended few filopodia in regions away from the vascular front (Fig. 3*C*). Quantification showed 75% more filopodia extensions at the vascular front, 125% more branchpoints within the vascular plexus, and an 80% increase in the area covered by vessels in *Dll4*$^{+/-}$ compared with wild-type (Fig. 3 *E–G*). This increase in the vessel coverage suggested that *Dll4*$^{+/-}$ vessels may also have proliferation defects. Quantification of BrdU-labeled (S-phase cells) or phospho-histone-H3-stained ECs (M-phase cells) indicated a modest (1.16-fold) non-significant increase in proliferating ECs in *Dll4*$^{+/-}$ vessels compared with wild-type (Fig. 3*H*). Taken together, these observations suggest that *Dll4*$^{+/-}$ retinal vessels are functional, and that the major defect in vessel patterning is due to inappropriate and excessive sprouting.

In addition to *Dll4*, other genes are also expressed at high levels in endothelial tip cells in the retina, including *Pdgfb*[23] and *unc5b*[26]. Com-

pared with wild-type, *Dll4*$^{+/-}$ retinal vessels expressed *Pdgfb* (Fig. 3 *I*, *J*, *M*, and *N*) and *Unc5b* (Fig. 3 *K*, *L*, *O*, and *P*), over an expanded area, especially in the hyperfused plexus. Thus, vessels from *Dll4*$^{+/-}$ retinas display genetic as well as morphological (filopodia) and behavioral (hyperfused vessels) indicators of an expansion in the number of ECs that have a "tip cell" phenotype, suggesting that Dll4 normally functions to suppress tip cell formation in growing vessels.

DLL4 IN TUMOR NEOANGIOGENESIS

As mentioned earlier, the high sensitivity of the embryonic vasculature to Dll4 levels raises the possibility that it may constitute a good target for intervention in adult neovascularization, both in proangiogenic as well as in anti-angiogenic settings, such as inhibiting tumor growth by targeting its vasculature.

To try to understand how Dll4 levels influence tumor vasculature, we studied vascular response and remodeling in tumors transplanted to adult *Dll4*$^{+/-}$ mutant and wild-type mice[27]. Mice received implants of S180 tumor cells. Tumor and adjacent tissue harvested after 10 days was examined for vascular response by PECAM, and α-SMA immunolocalization. Wild-type mice showed increased vascular response in the tumor (Fig. 4B) and the vessels displayed an organized network. In comparison, *Dll4*$^{+/-}$ mice showed an even greater increase in the vascular response (1.5-fold increase, $P < 0.05$). Furthermore, the vessels showed lack of architecture and loss of hierarchy. Thus vascular response was increased but maturation was lacking. Maturation of newly forming vessels accompanies the recruitment of pericytes. We hypothesized that newly forming vessels in *Dll4*$^{+/-}$ mice may be defective in pericyte recruitment. Thus localization of pericytes with α-SMA antibodies showed abundant signal in tumor vessels in wild-type mice, whereas tumor vessels in *Dll4*$^{+/-}$ mice showed a profound deficiency in pericyte coverage. Reduced recruitment of pericytes may contribute to the lack of vascular hierarchy observed in the tumor vessels of *Dll4*$^{+/-}$ mice. Furthermore, these findings reveal a novel function of *Dll4* in the recruitment of pericytes to newly forming vessels. We next wished to determine if defective vascular response in adult mice leads to alteration in gene expression, in particular *Dll4*. To this end, we used the *LacZ* reporter included in the targeting vector used to generate mutant mice to observe *Dll4* promoter activity. *Dll4*$^{+/-}$ mutant mice showed highly structured *LacZ*--expressing vessels in the normal tissue adjacent to the tumor, whereas LacZ activity was markedly increased in vessels within the tumor vessels (Fig. 4C), indicative of *Dll4* activation in the tumor vasculature. PECAM localization in serial sections of the tumor vessels was done to determine the extent of *Dll4* activation in tumor vasculature, showing that it is expressed in the majority but not in all tumor vessels.

CONCLUSION

Dll4 appears to act in endothelial cells in at least two different ways, establishing arterial endothelial cell fate in development and regulating the intensity of the angiogenic response. The potential to therapeutically modulate angiogenesis through this signaling pathway constitutes a promising research avenue. The available data suggest a model in which Dll4, expressed in endothelial tip cells, inhibits the angiogenic response of adjacent ECs to VEGF stimulation, most likely through Notch signaling. This mechanism would permit an asymmetric cellular response to VEGF stimulation during vascular sprouting by allowing some ECs to respond to a local VEGF gradient by forming a sprout, while, through upregulation of *Dll4* expression, inhibiting adjacent cells from also forming sprouts. When even a single *Dll4* allele is absent, or when Notch signaling is blocked, this suppression is lost, resulting in increased sprout formation

and tip cell filopodia. This mechanism provides an elegant negative feedback system intrinsic to ECs to control their response to VEGF and suggests that vascular network formation is coordinated by VEGF and Dll4/Notch signaling. In addition, Dll4 appears to be involved in pericyte recruitment and therefore, in the stabilization of newly formed vascular branches. We are currently testing the effect of modulators of vascular Notch signaling *in vivo* to address the feasibility of its use in therapeutic intervention in angiogenesis.

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LEUKEMIA CELLS AND THE BONE MARROW ENDOTHELIUM

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ABSTRACT

Angiogenesis, the recruitment and proliferation of endothelial cells leading to formation of new blood vessels from preexisting ones, plays a critical role in the growth of solid tumors. The biological and clinical implications of bone marrow (BM) endothelium interaction with hematological tumor cells remain controversial. However, there is evidence suggesting the existence of BM niches where endothelial and leukemia cells contribute with mutually beneficial stimuli that promote both an angiogenic phenotype and leukemia expansion. Factors such as SDF1 and VEGF have been implicated in the interplay between endothelium and leukemia, and may constitute targets for therapeutic intervention. Another factor, IL-7, is produced by BM stroma and endothelium, and appears to stimulate endothelial cells. Moreover, we and others have shown that IL-7 is a T-cell leukemia growth factor. IL-7 mediates leukemia proliferation and viability by triggering the activation of PI3K/Akt(PKB) pathway leading to $p27^{kip1}$ downregulation, Bcl-2 upregulation, and consequent cell cycle progression and decreased apoptosis. Given the evidence IL-7 can further stimulate leukemia T-cell motility and directional migration, it is tempting to hypothesize the existence of BM niches where stroma/endothelium-produced IL-7 promotes leukemia expansion.

Keywords: Leukemia; bone marrow endothelial cells; IL-7; angiogenesis

INTRODUCTION

Cancer cells are not isolated entities, multiplying inexorably irrespectively of what surrounds them. On the contrary, they evolve in complex microenvironments and are responsive to exogenous stimuli that include the extracellular matrix, cytokines, chemokines and cell-cell contact. In addition, they are able to modulate their environment in an advantageous manner, for example by releasing defined soluble factors, contributing to extracellular matrix remodeling, and stimulating or inhibiting neighboring cells.

A TUMOR'S NEED FOR ANGIOGENESIS

The recruitment and proliferation of endothelial cells from preexisting vasculature resulting in the formation of new blood vessels defines angiogenesis. Tumors, despite their tendency to expand relentlessly, are nonetheless constituted by cells and thus restricted by two basic needs, which no cell is able to overcome, malignant as it may be: oxygen and nutrients. As the tumor mass grows, how do the cells in the inner portions of the tumor satisfy these essential needs, since passive diffusion is not sufficient to deliver oxygen and nutrients and remove metabolic waste? The answer is known for many years now: they release proangiogenic factors, which promote the formation of new vasculature that penetrates into the tumor mass[1]. More precisely, there is evidence that production of proangiogenic factors by hypoxic cells in the tumor core overcomes the expression of antiangiogenic factors in what is called the 'angiogenic switch', which will ultimately shift the balance towards an angiogenic phenotype[2] (Figure 1). Evidently, the dependency on angiogenesis for tumor growth has stimulated the quest for anti-angiogenic therapeutic agents and strategies involving the use of inhibitors of angiogenesis with the ultimate goal of rendering cancer a controlled, chronic 'disease'[2,3]. However, much is still to be learnt, since tumors often appear to be able to circumvent the effect angiogenesis inhibitors[4].

HOW DO TUMORS PROMOTE ANGIOGENESIS?

Upon the angiogenic switch, the net balance of proangiogenic factors perceived by endothelial cells (ECs) will result in their activation. Activated cells loosen the contacts with adjacent cells and start pro-

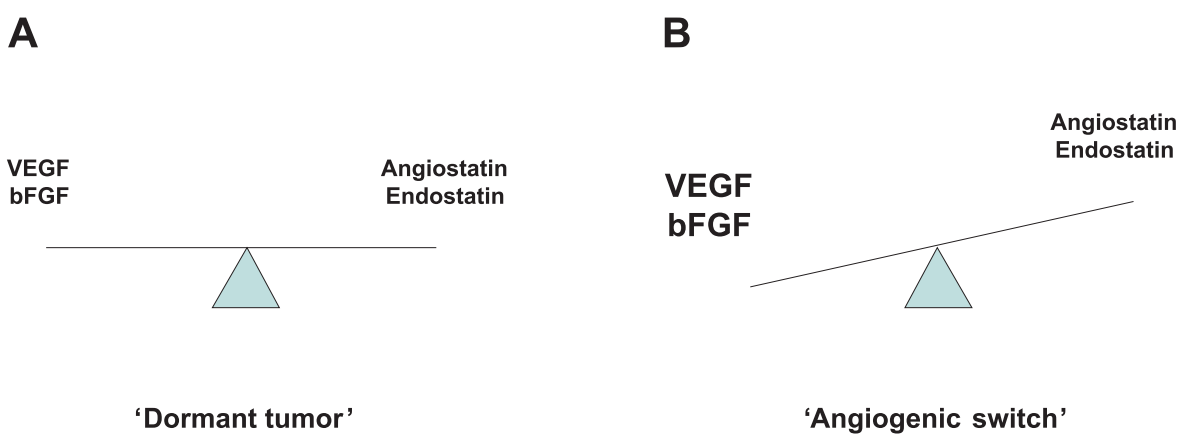

Fig. 1 – Schematic simplified representation of the "angiogenic switch". **(A)** Dormant tumors present a balance between the levels of proangiogenic factors (represented here by VEGF and bFGF) and antiangiogenic factors (represented here by angiostatin and endostatin). **(B)** Upon increase in hypoxic conditions tumors tend to increase the production of proangiogenic factors and/or block the synthesis of antiangiogenic molecules. The "angiogenic switch" occurs when the net signal received by nearby endothelium is angiogenic.

ducing proteases (including matrix metalloproteases – MMPs) that locally degrade the basement membrane. Subsequently, ECs are able to move through the gap in the basement membrane and into the extracellular matrix (ECM). Neighboring ECs may subsequently follow the leading cells into the ECM[5]. After extravasation, ECs continue to secrete proteases, which also degrade the ECM, allowing the ECs to move away from the parent vessel and towards the tumor, forming sprouts that eventually originate capillary structures with a lumen, form anastomoses, and allow for actual blood flow. To do so, ECs respond to the proangiogenic factors first by migrating in a chemotactic-like fashion and second by proliferating.

ANGIOGENESIS IN HEMATOLOGICAL TUMORS?

It is rather intuitive that solid tumors should rely on promoting angiogenesis to grow. However, why would hematological tumors develop such an approach, since in general they do not assemble into masses of malignant cells? The surprising answer is that most of them do. For example, the leukemic bone marrow (BM) is commonly characterized not only by a significant infiltration with malignant blasts that disrupt the normal BM architecture but also by increased numbers of endothelial cells and blood vessels. This is true for both myeloid and lymphoid leukemia[3,6]. Interestingly, there appears to be a clear correlation between the number of leukemic blasts and the number of vessels in the BM[7,8], hinting on the existence of a crosstalk and interdependency between BM endothelial cells (BMECs) and leukemia cells.

INTERPLAY BETWEEN LEUKEMIA AND ENDOTHELIAL CELLS IN THE BONE MARROW

What are the factors involved in the interplay between leukemia cells and BMECs? It is known that hematological tumors produce VEGF, bFGF and other proangiogenic factors[3,9], and their BM and plasma levels are usually elevated in patients as compared to normal controls[9,10]. Similarly to what happens in solid tumors, these factors are implicated in angiogenesis in the BM of leukemia patients[11]. In turn, BMECs were shown to produce leukemia-stimulatory agents. One of these factors, SDF-1/CXCL12 is a potent chemoattractant for CXCR4-expressing leukemia cells[12], and exemplifies the capacity that BMECs also have to modulate the function of tumor cells. Evidently, VEGF and SDF-1 are not the only players in the leukemia-BMEC crosstalk and identification of other molecules with an impact on these processes should have great therapeutic potential.

INTERLEUKIN-7

The BM stroma produces interleukin 7 (IL-7), which promotes T-cell acute lymphoblastic leukemia (T-ALL) cell viability and proliferation[13]. The oncogenic potential of IL-7 was shown several years ago by experiments with IL-7 transgenic mice that demonstrated that these animals developed B and T-cell neoplasms[14]. Subsequently, we demonstrated that IL-7 promotes both T-ALL cell viability and cell cycle progression by downregulating the cyclin-dependent kinase inhibitor $p27^{kip1}$. Decreased expression of $p27^{kip1}$ contributes to cyclin-dependent kinase activity, Rb hyperphosphorylation and progression towards S and G2/M phases of the cell cycle. In addition, $p27^{kip1}$ downregulation contributes to Bcl-2 upregulation, which is mandatory for IL-7-mediated survival of T-ALL cells[15]. These downstream effects are dependent on activation of PI3K/Akt(PKB) signaling pathway[16] (Figure 2).

Irrespectively of the associated molecular mechanisms, IL-7 is a clear *in vitro* growth factor for T-ALL cells and our most recent studies indicate that IL-7 may significantly contribute to human leukemia progression *in vivo* (Silva et al, unpublished data). Thus, it is interesting to speculate whether IL-7 might also play a role on the crosstalk between leukemia cells and BMECs. The answer is not yet known. How-

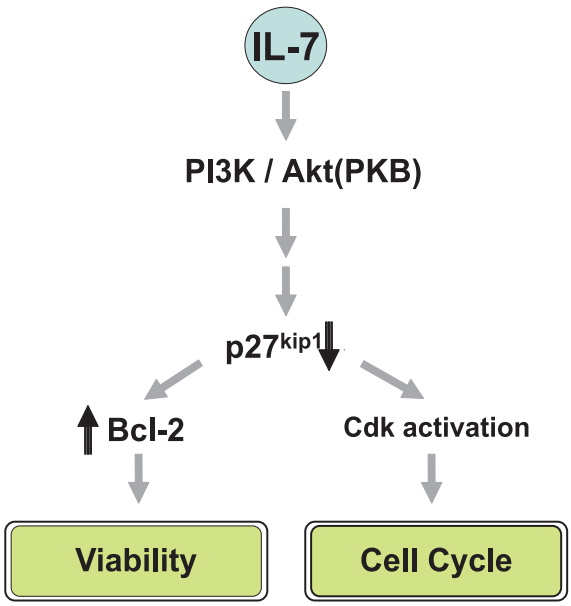

Fig. 2 – Summarized molecular and functional effects of IL-7 on leukemia T-cells.

ever, BMECs appear to express IL-7Rα and secrete IL-7 (Andres Yunes et al, unpublished data), suggesting the possibility of an autocrine/paracrine stimulatory loop (Figure 3). Stimulation with IL-7 prevents BMEC cell death in medium without growth factors, and potentiates the proliferative effect of proangiogenic factors. Finally, IL-7 contributes to the formation of capillary-like structures in matrigel-cultured BMECs (Andres Yunes et al, unpublished data).

As stated above, tumor cells including leukemias, produce angiogenic factors that stimulate BMEC migration. Likewise, it is currently known that BMECs are also able to attract leukemia cells in vitro[10] and in vivo[12]. Evidently, there are several candidates as mediators of this effect, the most prominent of which is SDF-1[17]. However, it is possible that IL-7 might also contribute to this effect in particular niches. Our preliminary data indicate that IL-7 promotes T-ALL cell motility and directional migration in vitro (Henriques et al, unpublished data). Hence, IL-7 produced by BMECs could not only participate in the stimulation of BMECs themselves but also in the recruitment of leukemia cells, with consequent stimulation of their viability and proliferation. In turn, it is known that IL-7 stimulates VEGF production at least in some cells, including normal thymocytes and breast cancer cells[18]. This raises the possibility that IL-7 stimulation of leukemia cells will further contribute to the production of VEGF that will serve as a growth factor for both leukemia cells and BMECs[11]. The model formulated from the studies and hypotheses described here is presented in Figure 3.

CONCLUSIONS

In summary, evidence arising from different studies indicates that there is a clear interplay between leukemia cells and BMECs that eventually leads to a positive loop, which contributes to increased angiogenesis and tumor expansion. Whether such a loop includes a relevant role for the BM microenvironmental cytokine IL-7 remains to be elucidated, but it is tempting to hypothesize the existence of BM niches where

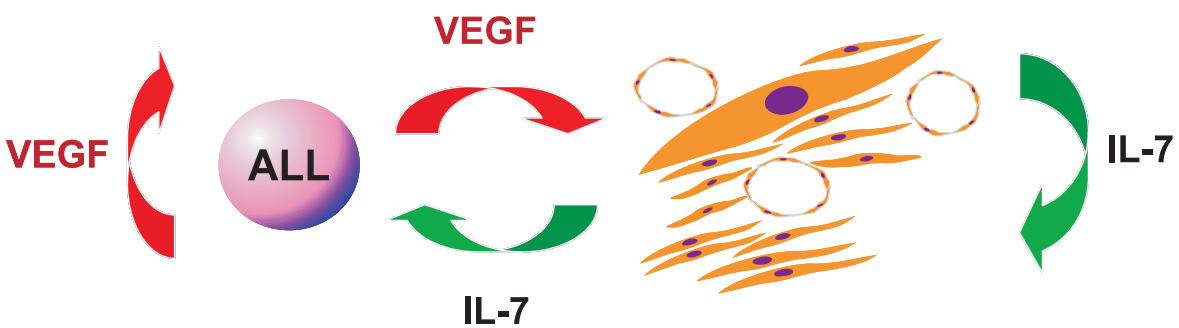

Fig. 3 – Hypothetical model of the contribution of IL-7 to endothelium – leukemia interactions in the BM.

stroma and/or endothelium-produced IL-7 participates in leukemia growth. Future studies should address this question and explore the possibility of targeting it for therapeutic purposes.

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COMPUTATIONAL HEMORHEOLOGY: PROGRESS IN BLOOD COAGULATION MODELLING

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ABSTRACT

In this paper we present a brief description of the physical properties of blood and its formed elements followed by a short overview of some constitutive models that can mathematically characterize blood rheology and be used in computer simulations. In particular, preliminary numerical results obtained for a comprehensive model of blood coagulation and clot formation, that integrates physiologic, rheologic and biochemical factors will be presented and discussed.

Key-words: blood rheology, coagulation, shear-thinning flows, numerical simulations, finite--volumes.

INTRODUCTION

Blood is a concentrated fluid suspension of multiple deformable cells with complex rheological characteristics. Blood performs the essential functions of delivering oxygen and nutrients to all tissues, it removes waste products and defends the body against infection through the action of antibodies. The blood circulation in the cardiovascular system depends not only on the driving force of the heart and the architecture and mechanical properties of the vascular system, but also on the rheology and flow properties of blood itself. Hemodynamic factors such as flow separation, flow recirculation, or low and oscillatory wall shear stress are recognized as playing an important role in the localization and development of arterial diseases. However, the composition and

the physical properties of blood constituents are essential to analyze and model the circulation.

Hemorheology is the science that studies the flow properties of blood and its formed elements and the relationship to normal and abnormal physiology. This field involves the investigation of the macroscopic behaviour of blood determined in rheometric experiments, its microscopic properties *in vitro* and *in vivo* and studies of the interactions among blood cellular components and between these components and the endothelial cells that line blood vessels. Advances in hemorheology have contributed in particular to the fundamental understanding of the changes in the rheological properties of blood and its components due to pathological disturbances and are based on the evidence that they might be the primary cause of many cardiovascular diseases.

The interactions between hemorheological factors and hemodynamical mechanisms are highly complex and the role of blood rheology in physiopathological processes is still not well understood. Therefore the mathematical and numerical study of powerful, yet simple, constitutive models that can capture the rheological response of blood over a range of physiological flow conditions is ultimately recognized as an important tool for clinical diagnosis and therapeutic planning.

The aim of this paper is to present a brief description of the physical properties of blood, including its non-Newtonian characteristics, and review some of the continuum mathematical models of blood rheology that have been proposed in the literature and can be used in computer simulations. A discussion of some preliminary numerical results obtained for a physiological meaningful model of blood coagulation and clot formation, based on the model introduced in[3] will also be included in this paper.

PHYSICAL PROPERTIES OF BLOOD

Whole blood is a concentrated suspension of formed cellular elements that includes red blood cells (RBCs) or erythrocytes, white blood cells (WBCs) or leukocytes and platelets or thrombocytes. These cellular elements are suspended in an aqueous polymeric and ionic solution of low viscosity, the plasma, containing electrolytes and organic molecules such as metabolites, hormones, enzymes, antibodies and other proteins. The formed elements represent approximately 55% by volume of the normal human blood. The process by which all formed elements of the blood are produced (hematopoiesis), occurs mostly in the bone marrow, where cells mature from a primitive stem cell. Important factors in regulating blood cell production include the environment of the bone marrow, interactions among the cells, and secreted chemicals called growth factors[7,8].

Plasma (separable by centrifugation) consists primarily of water (approximately 90-92% by weight) in which inorganic and organic substances (approximately 1-2%) and various proteins (fibrinogen, prothrombin, tissue-factor, albumin, lipoproteins, immune proteins, etc.) are dissolved along with various ions. Plasma's physiological function is the transport of nutrients and wastes throughout the body.

RBCs are highly flexible biconcave discoid particles, without nuclei, typically 6-8 μm in diameter, with a very thin membrane (of maximum thickness 50-100 angstroms[15]) consisting of proteins (spectrin) and lipids. They are filled with a fluid, which is an almost saturated solution (approximately 32% by weight) of hemoglobin, 65% of water, the remainder being other proteins, lipids, adenosine diphosphate (ADP), adenosine triphosfate (ATP) and ions. Hemoglobin is the protein inside red blood cells that gives blood its red color and is primarily involved in oxygen and carbon dioxide transport between the lungs and the living tissues of the body. Erythrocytes are the most numerous of the formed elements (about 98%) and have the largest influence on the mechanical properties of blood. The volume concentration of RBCs in whole blood is called the hematocrit (Ht). The normal values of hematocrit in

humans range from 40-45%, for adults in normal health conditions, to 55-68% for newborns. These values depend on several factors including age, sex of the individuals or health conditions and have a strong influence on the rheology of blood, in particular on blood viscosity.

WBCs, which are much less numerous than erythrocytes (less than 1% of the volume of blood), are normally roughly spherical in shape with diameters ranging from about 7-22 μm. They have nuclei and are composed of five morphologically different cell types: basophils, eosinophils and neutrophils (collectively called granulocytes) and also monocytes and lymphocytes. Leukocytes circulate in the blood stream and when activated by inflammation or by the presence of foreign organisms, change their rheological properties such as deformability. This activation generates an inner force governing a complex cascade of rolling along the vessel wall, induces adhesion to the microvascular endothelium and, ultimately, leads to extravascular migration into the surrounding tissue. However, these processes are believed to have little influence on the rheology of blood, except in extremely small vessels like capillaries or in disease conditions, e.g.[19]. Leukocytes play a key role in the immune system, namely in the protective mechanisms of the body against diseases, in tissue inflammation and in other physiologic and pathological processes, related to early stages of development of cardiovascular diseases. The study of the mechanics of leukocytes is therefore of great interest, see e.g.[12,13,17,32].

Platelets, small discoid non-nucleated cell fragments containing various chemicals such as serotonin, thrombin, and ADP, are much smaller than erythrocytes (approximately 6 μm^3 in size as compared to 90 μm^3) and form a small fraction of the particulate matter in human blood (around 3% by volume). Due to several biochemical reactions and mechanical processes related to prolonged exposure to high shear stress or rapid increases in shear stress, platelets can be activated and become involved in the formation of clot cascades and blood coagulation. If blood is allowed to clot, the remaining fluid is called serum, which is similar to plasma but is missing the protein fibrinogen. Of all the components of blood, platelets are by far the most sensitive to chemical and physical agents and play a critical role in the coagulation process as will be briefly discussed in the next Section.

PLATELET ACTIVATION AND BLOOD COAGULATION

Hemostasis is a complex physiological process involving an interaction between blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolytic proteins. When blood coagulates in a blood vessel during life, the process is called thrombosis. Hemostasis keeps blood flowing while allowing solid clot formation, or thrombosis, to prevent blood loss from sites of vascular damage. The hemostatic system preserves intravascular integrity by achieving a balance between hemorhage and thrombosis.

Blood platelets participate in both hemostasis and thrombosis. The first stage of thrombogenesis is platelets activation, followed by platelets aggregation, adhesion and blood coagulation, with the formation of clots. Blood platelets can be activated by prolonged exposure to high or rapid increase in shear stress that lead to erythrocytes and platelets damage. This is due to mechanical vascular injuries or endothelial dysfunction, alterations in the blood composition, fissuring of atherosclerotic plaques as well as to the contact of blood with the surfaces of medical devices. Numerous experimental studies recognize that clot formation rarely occurs in regions of parallel flow, but primarily in regions of stagnation point flows, within blood vessel bifurcations, branching and curvatures.

Following endothelial disruption, there is an immediate reflex that promotes vasoconstriction, minimizing vessel diameter and diminishing blood

loss. Vasoconstriction slows blood flow, enhancing platelet adhesion and activation. During activation platelets undergo intrinsic and extrinsic mechanisms leading to a series of chemical and morphological changes. Organelles contained in the platelet cytoplasm bind to collagen (exposed by arterial damage), release their contents of cytoplasmic granules containing serotonin, adenosine diphosphate (ADP) and platelet-activating factors and platelets become spheroids in shape. Additional platelets attracted by ADP are activated, interact with plasma proteins like fibrinogen and fibrin and promote platelet aggregation and adhesion to sub-endothelial tissue. This results in the formation of hemostatic plugs and concludes the primary hemostasis. However, when the concentration of activators exceeds a certain value, platelet aggregates that are formed by this process can break up, damaging the platelets and causing aggregation at locations other than at the site of damage.

The final hemostatic mechanism or secondary hemostasis is coagulation. The biochemical process leading to clot formation involves a very complex cascade of enzymatic reactions. Thrombin is the bottom enzyme of the coagulation cascade. Prothrombin activator converts prothrombin to thrombin. Thrombin activates platelets that release ADP which lead in turn to the activation of other platelets. It converts fibrinogen, a blood protein, into polymerized fibrin, stabilizing the adhered platelets and forming a viscoelastic blood clot (or thrombus) (e.g. [27,31]).

The clot attracts and stimulates the growth of fibroplasts and smooth muscle cells within the vessel wall, and begins the repair process which ultimately results in fibrinolysis and in the dissolution of the clot (clot lysis). Clot dissolution can also occur due to mechanical factors such as high shear stress[27]. In practice a blood clot can be continuously formed and dissolved. Generally, many factors affect its structure, including the concentration of fibrinogen, thrombin, albumin, platelets and red blood cells and other not specified factors which determine cross-linked structure of the fibrin network. At the end of the hemostatic process, normal blood flow conditions are restored. However, some abnormal hemodynamic and biochemical conditions of flowing blood, related to inadequate levels or dysfunction of the hemostatic system, may lead to pathologies like thromboembolic or bleeding disorders of great clinical importance.

The mechanism of platelet activation and blood coagulation is quite complicated and not yet completely well understood. Recent reviews detailing the structure of the blood coagulation system are available for example in [3,31].

BLOOD CONSTITUTIVE MODELLING

The study of blood flow in the vascular system is complicated in many respects and thus simplifying assumptions are often made.

Plasma behaves as a Newtonian fluid but whole blood has non-Newtonian properties. In the large vessels where shear rates are high enough, it is also reasonable to assume that blood has a constant viscosity and a Newtonian behaviour. However in smaller vessels, or in some diseased conditions, the presence of the cells induces low shear rate and whole blood exhibits remarkable non-Newtonian characteristics, like shear-thinning viscosity, thixotropy, viscoelasticity and possibly a yield stress. In particular, at rest or at low shear rates, blood seems to have a high apparent viscosity (due to RBCs aggregation into clusters called *rouleaux*) while at high shear rates the cells become disaggregated and deform into an infinite variety of shapes without changing volume (deformability of RBCs), resulting in a reduction in the blood's viscosity. The deformed RBCs align with the flow field and tend to slide upon plasma layers formed in between. Attempts to recognize the shear-thinning nature of blood were initiated by Chien et al.[10,11] in the 1960s. Empirical models like the power-law, Cross, Carreau or W-S generalized Newtonian fluid models (see, [5,37]) have been obtained by fitting experimental data in one

dimensional flows. Recently, Vlastos et al.[36] proposed a modified Carreau equation to capture the shear dependence of blood viscosity.

Experiments on blood at low shear rates are extremely difficult to perform and consequently a controversy remains on the behavior of blood at the limit of zero shear rate, leading researchers to believe in the existence of a critical value of stress (yield stress) below which blood will not flow. The treatment of yield stress as a material parameter should be independent of experimental factors and of yielding criteria and this is not the case for blood. In fact there exists a large variation in yield stress values for blood reported in the literature (e.g [22]). The finite time required for the changes in blood microstructure is related to blood yield stress and thixotropy. Charm et al.[9] found that Casson's model gives the best fit to blood data. Casson's and Herschel-Bulkley models[30] are generalizations of the Bingham model that can capture both the yield stress and the shear thinning behavior of blood.

None of these homogeneized models are capable of describing the viscoelastic response of blood. Blood cells are essentially elastic membranes filled with a fluid and it seems reasonable, at least under certain flow conditions, to expect blood to behave like a viscoelastic fluid. At low shear rates RBCs aggregate and are 'solid-like', being able to store elastic energy that accounts for the memory effects in blood. Dissipation is primarily due to the evolution of the RBC networks and, given the paucity of data on temperature effects, the internal energy is assumed to depend only on the deformation gradient. At high shear rates, the RBCs disaggregate forming smaller rouleaux, and later individual cells, that are characterized by distinct relaxation times. RBCs become 'fluid-like', losing their ability to store elastic energy and the dissipation is primarily due to the internal friction. Upon cessation of shear, the entire *rouleaux* network is randomly arranged and may be assumed to be isotropic with respect to the current natural configuration. Thurston (see [34]) was among the earliest to recognize the viscoelastic nature of blood and that the viscoelastic behaviour is less prominent with increasing shear rate. He proposed a generalized Maxwell model that was applicable to one dimensional flow simulations and observed later that, beyond a critical shear rate, the non-linear behaviour is related to the microstructural changes that occur in blood (see [35]). Recently an approximate model inspired on the behaviour of transient networks in polymers and exhibiting shear-thinning, viscoelasticity and thixotropy, related to the microstructure of blood, has been derived by Owens[21].

Other rate type constitutive models for describing blood rheology have been proposed in the recent literature. Yeleswarapu[39] has obtained a three constant generalized Oldroyd-B model by fitting experimental data in one dimensional flows and generalizing such curve fits to three dimensions. It captures the shear-thinning behavior of blood over a large range of shear rates but it has its limitations, given that the relaxation times do not depend on the shear rate, which does not agree with experimental observations. The model developed by Anand and Rajagopal[1] in the general thermodynamic framework of Rajagopal and Srinivasa[25] includes relaxation times depending on the shear rate and gives good agreement with experimental data in steady Poiseuille flow and oscillatory flow.

Continuum models for blood flow are very important (see the recent reviews [28,29,33]) but they are not appropriate in the capillary network (see, e.g. Popel and Johnson[23] and Pries and Secomb[24] for an overview of hemorheology in the microcirculation).

It is now recognized the increasing importance of considering phenomena at the molecular scale where interactions between individual proteins are relevant and an enormous variety of biochemical and biological phenomena at the cells level are influenced and even controlled by fluid dynamic forces[16]. As a consequence, new insights emerged into the pathogenesis of diseases as in the case of atherosclerosis, or into the prevention of important physiological processes like thrombus formation in surgical patients.

NUMERICAL SIMULATIONS OF A BLOOD COGULATION MODEL

While there has been a considerable research effort in blood rheology, the constitutive models have thus far focused on the aggregation and deformability of the RBCs, ignoring the role of platelets in the flow characteristics. In the last two decades mathematical modelling and computer simulation research has emerged as a useful tool, supplementing experimental data and analysis and giving new insights in the studies of the regulation of the coagulation cascade, in clinical applications and device design. Reliable phenomenological models that can predict regions of platelet activation and deposition (either in artificial devices or in arteries) have the potential to help optimize design of such devices and also identify regions of the arterial tree susceptible to the formation of thrombotic plaques and possible rupture in stenosed arteries. Most of the models that are currently in use neglect some of the biochemical or mechanical aspects involved in the complex phenomena of blood coagulation and must be considered as first approaches to address this oversight, see for example [14,18,38]. M. Anand, K. Rajagopal and K. R. Rajagopal[2,3] recently developed a phenomenological comprehensive model for clot formation and lysis in flowing blood that extends existing models to integrate biochemical, physiologic and rheological factors. In what follows we present some preliminary numerical results for a simplified version of this model. A detailed description of these results can be found in [6].

Governing Equations

A generalized Newtonian model with shear--thinning viscosity has been adopted for describing the flow of blood. We denote by ***u****(x,t)* and *p(x,t*) the blood velocity and pressure in the domain *Ω*, the vascular lumen, with $t \geq 0$. The application of the physical principles of momentum and mass conservation for an incompressible viscous fluid leads to the equations defined in *Ω*

$$\rho\frac{\partial \mathbf{u}}{\partial t} + \rho(\mathbf{u}\cdot\nabla)\mathbf{u} + \nabla p - \operatorname{div}\boldsymbol{\tau}(\boldsymbol{u}) = 0 \quad \textbf{(1)}$$

$$\operatorname{div}\mathbf{u} = 0$$

completed with appropriate initial and boundary conditions. Here ρ is the fluid density and $\tau(u)$ is the deviatoric stress tensor, proportional to the symmetric part of the velocity gradient, given by

$$\boldsymbol{\tau}(\boldsymbol{u}) = \mu(\dot{\gamma})(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)$$

where $\dot{\gamma} = \sqrt{\frac{1}{2}(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T) : (\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)}$ is the shear rate (a measure of the rate of shear deformation) and $\mu(\dot{\gamma})$ is the shear dependent viscosity function which decreases with increasing shear rate. The generalized Cross model has frequently been used for blood. The corresponding viscosity function is written as

$$\mu(\dot{\gamma}) = \mu_\infty + \frac{\mu_0 - \mu_\infty}{(1 + (\lambda\dot{\gamma})^m)^a} \quad \textbf{(2)}$$

where $\mu_0 = \lim_{\dot{\gamma}\to 0}\mu(\dot{\gamma})$ and $\mu_\infty = \lim_{\dot{\gamma}\to\infty}\mu(\dot{\gamma})$ are the asymptotic viscosities at low and high shear rates. Using nonlinear regression analysis, it is possible to fit viscosity functions against blood viscosity experimental data and obtain the corresponding parameters. However, blood viscosity is quite sensitive to a number of factors including hematocrit, temperature, plasma viscosity, age of RBCs, exercise level, gender or disease state, and care must be taken in selecting blood parameters for blood flow simulations. Here we have adopted the following material constants (taken from [20]):

$$\begin{array}{ll} \mu_0 = 1.6\times 10^{-1} Pas & \mu_\infty = 3.610^{-3} Pas \\ a = 1.23, m = 0.64 & \lambda = 8.2s \end{array} \quad \textbf{(3)}$$

The viscosity function (2) with values (3) is represented in Fig. 1.

Our model (see [3]) includes not only rheological factors but also biochemical indicators that are essential to describe coagulation and fibrino-

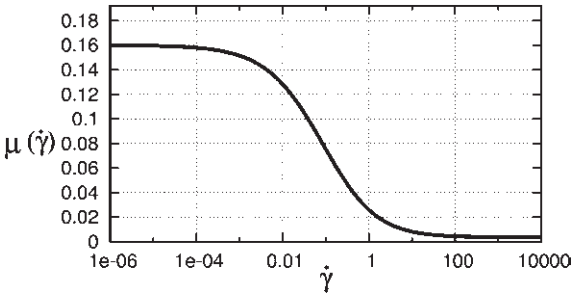


Fig. 1 – Variation of the apparent viscosity as a function of the shear rate for the generalized Cross model with physiological parameters for blood given by (3) (from [20])

lysis dynamics and consequently the formation, growth and dissolution of clots. A set of coupled advection-diffusion-reaction equations modelling the evolution in time and space of various enzymes, proteins and platelets involved in the extrinsic pathway of coagulation process, takes the following form

$$\frac{\partial C_i}{\partial t} + \mathrm{div}(C_i \boldsymbol{u}) = \mathrm{div}(D_i \nabla C_i) + R_i, \quad i = 1, \ldots, 23 \qquad \textbf{(4)}$$

In these equations C_i stands for the concentration of the i-th reactant, D_i denotes the corresponding diffusion coefficient (which could be a function of the shear rate) and $\boldsymbol{u}$ is the velocity field. R_i are the non-linear reaction terms that represent the production or depletion of C_i due to the enzymatic cascade of reactions. The specific form of the reaction terms and diffusion coefficients for the 23-model equations (4) can be found in [3] (see also [6]).

Equations (4) are complemented with appropriate initial and flux boundary conditions involving the concentration of the various species at the inner wall that reflect the injury to the blood vessel.

Under normal conditions no clot exists and we assume that the presence of all these constituents does not affect the velocity of the bulk flow. Clot formation occurs in the vicinity of the injured wall, when an activation threshold in the flux boundary conditions (related to the appearance of tissue factor TF-VIIa complex) is exceeded and the clotting cascade is initiated, which results in the increase of fibrin concentration in the clotting area. Clot growth is determined by tracking in time the extent of the flow region where fibrin concentration C_F equals or exceeds a specific critical value C_{Clot}. Clot dissolution occurs in regions where fibrin concentration drops below C_{Clot}, after initially exceeding it, or when the shear stress exceeds a certain critical value forcing clot's rupture. An important assumption of the model is to assume that the constitutive model for blood and clot are similar, but the material modulus has different values, in particular, the viscosity of the clot is 100 times higher than the viscosity of blood. The main features of the modelling approach are schematically shown in Fig. 2.

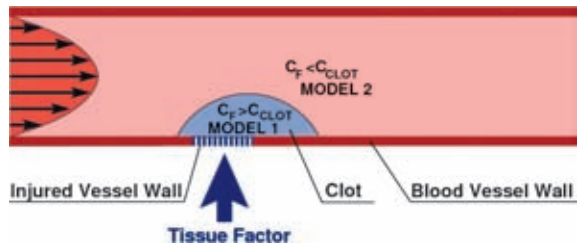


Fig. 2 – Clot modelling

Numerical Results

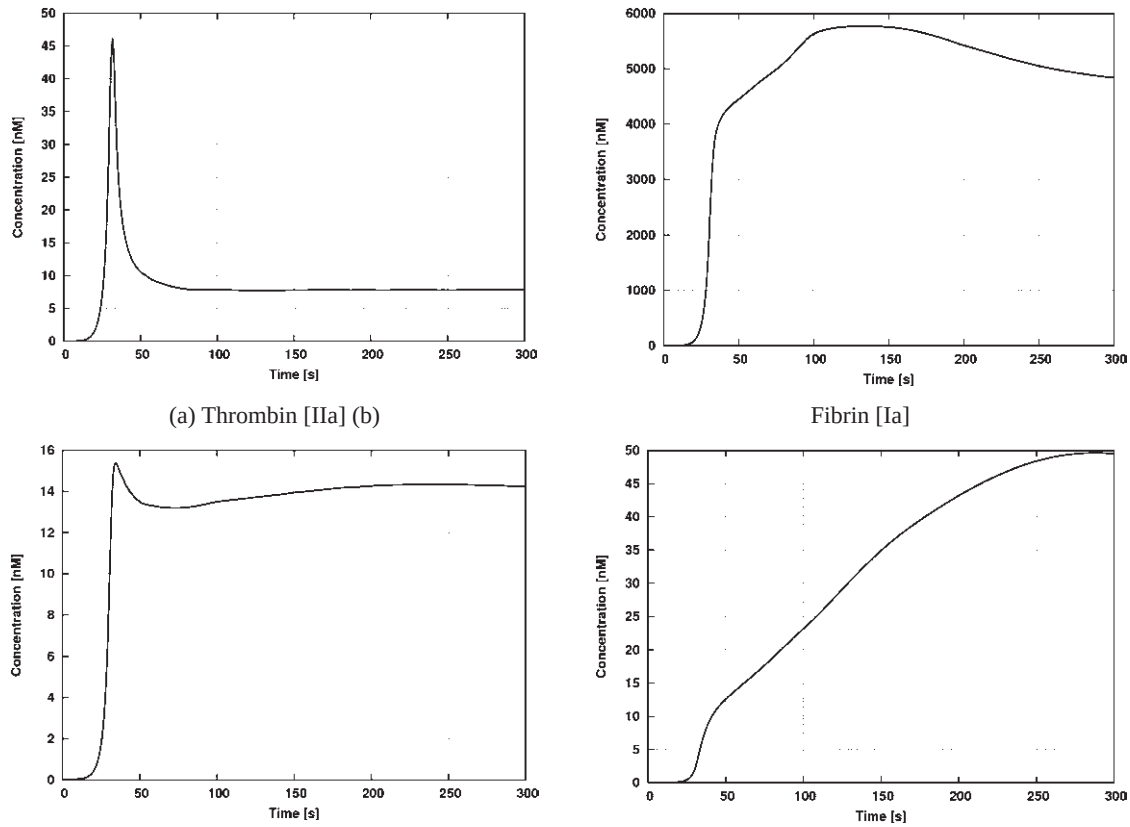
The numerical solution of the coupled fluid--biochemistry model was obtained using an original code based on a finite volume semi-discretization in space, on structured grids, and a simplified multistage time integration scheme. First, the velocity field is computed using equations (1) with a given viscosity, and the concentrations (including that of fibrin) are computed trough equations (4). The local viscosity is updated by a factor which depends on the local fibrin concentration in the clotting area and the velocity field is recomputed using the updated viscosity.

In this section we present preliminary results in a computational domain consisting of a segment of

a rigid-walled cylindrical vessel with diameter *6.2mm* and length *31mm*. A fully developed velocity profile (with mean velocity *3.1cm/s*) is prescribed at the inlet and homogeneous Neumann conditions with fixed pressure are imposed at the outlet. On the vessel wall no-slip Dirichlet conditions for the velocity field are enforced. In addition, we require normal physiological values prescribed as initial conditions for the concentrations of all chemical species (see [2,3,6]). The concentration boundary conditions, for all species, are set as homogeneous Neumann conditions (i.e. no flux) on the healthy vessel wall. In the injured wall region no flux boundary conditions are prescribed for all constituents except for seven species which are directly involved in the initiation of the coagulation cascade[2,3,6].

All the 23 chemical species play an important role in the clotting process. Their concentrations are computed pointwise in the whole computational domain. Figure 3 illustrates the evolution in time *(300s)* of four different concentrations, in the centre of the clotting surface. In particular, we observe that fibrin concentration increases rapidly and reaches its maximum value approximately *120s* after the initiation of the clotting cascade, remaining relatively stable after that time.

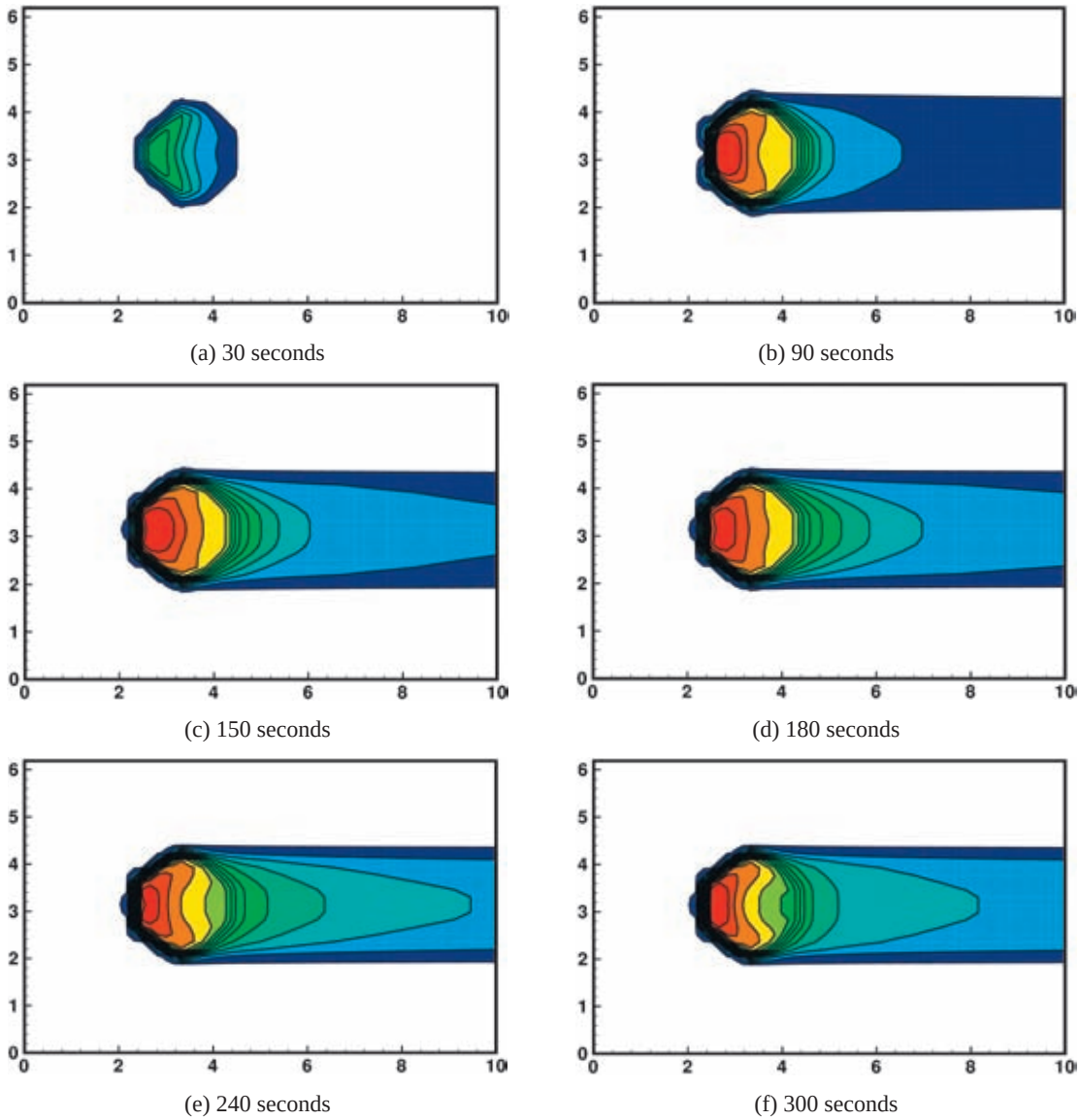
Clot growth can be better observed in Fig.4 which shows surface fibrin concentration contours in the time period *0 – 300s* of clotting. The length scales of both axes correspond to the axial and tangential coordinates, normalized by the vessel cross-section radius. Due to advection, fibrin is transported downstream on the injured vessel wall region and the clot's shape changes its form during the clotting process.



(a) Thrombin [IIa] (b) Fibrin [Ia]

(c) Tissue Plasminogen Activator [tPA] (d) Plasmin [PLA]

Fig. 3 – Time evolution of the concentrations of selected chemical species in the centre of the clotting surface



(a) 30 seconds (b) 90 seconds

(c) 150 seconds (d) 180 seconds

(e) 240 seconds (f) 300 seconds

Fig. 4 – Fibrin concentrations on the vessel wall during the first 5 minutes of clotting

Clot growth and dissolution needs more computational time and is beyond the purpose of the preliminary numerical simulations obtained with this model.

DISCUSSION

Preliminary numerical results of three--dimensional simulations for a simplified version of a model of clot growth and lysis, based on [2,3] and detailed in [6], have been presented here.

The inclusion of additional chemical constituents and their interactions, as those involved in platelets activation and aggregation, should be incorporated into the model to obtain more realistic results. Moreover, the blood flow model used in the simulations only captures its shear-thinning viscosity and could be improved using more complex rheological models. It would be interesting

to incorporate such extensions in the used solvers to obtain numerical results for a more realistic coagulation model that fits physiological experimental data and may be used in clinical applications. This is the object of our current research.

ACKNOWLEDGEMENTS

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MECHANISMS THAT REGULATE CELL TURNOVER OR TRANSFORMATION IN THE BONE MARROW MICROENVIRONMENT

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The Bone marrow (BM) is the major hematopoietic organ in adulthood, exists in the central bone cavities of long and axial bones. Marrow spaces form a trabecular structure with stromal cells, hematopoietic and endothelial progenitors. In long bones, one or two veins enter the marrow cavity and in flat bones there are several blood vessels with different sizes. Myelinated and non-myelinated nerves constitute BM enervation. Hematopoietic tissue consists in a various type of mature blood cells and their precursors.

Bone marrow microenvironment consists in BM stroma cells and factors, growth factors and cytokines, provided from stroma and blood vessels cells. Stroma cells have been considered the responsible in maintaining BM microenvironment but vasculature is also important because it is the oxygen supplier and exerts other functions (see below). BM stroma consists in different types of cells: endothelial cells, macrophages, adypocytes, fibroblasts, osteoblasts and extracellular matrix elements like binding proteins and fibronectin. For hematopoiesis to take place it is necessary a stable microenvironment that produces/expresses factors suitable for their migration, differentiation and lineage commitment.

ENDOTHELIAL CELLS IN THE BM MICROENVIRONMENT

Similar to what is seen in other organs, endothelial cells (EC) exert crucial functions within the BM microenvironment, namely by modulating the trafficking and the terminal differentiation of hematopoietic cells. Several studies have focused on the identification and function of adhesion molecules and chemokines secreted by bone marrow endothelial cells (BMEC), including ICAM-1, E-selectin (adhesion molecules), SDF-1 (chemokines) among others; this way, BMEC have been shown to exert a crucial role in cell trafficking in and out of the BM microenvironment. Subsequent studies have exploited these properties in transplant settings, for instance.

FACTORS THAT PROMOTE ENDOTHELIAL TURNOVER WITHIN THE BM MICROENVIRONMENT

Angiogenic growth factors such as Vascular endothelial growth factor (VEGF) promote the survival and modulate the hematopoietic-supporting

functions of BM endothelia. Conversely, abnormal production of VEGF within the bone marrow may promote endothelial proliferation, and consequently affect the hematopoietic microenvironment.

The expansion of endothelial cells within the BM microenvironment may thus provide a source of nutrients and oxygen, needed for a transformed and highly proliferative leukaemia clone/s. Therefore, similar to solid tumor growth, activation of the angiogenic program within the BM is obviously critical for the progression of leukemias, and bone marrow diseases in general.

More recently, we have tested the hypothesis that BMEC turnover/apoptosis might condition BM function and could have a crucial role in BM carcinogenesis. First, we studied the importance of TNF-alpha, which is abundantly secreted in the BM microenvironment and has the capacity to induce hematopoietic and vascular cell apoptosis (depending on the dose). Interestingly, TNF-alpha levels increase and show a remarkable correlation with BM recovery following irradiation (Figure 1). In vitro experiments have shown that blocking TNF-alpha in total BM cell cultures decreases apoptosis incidence, most remarkably in the endothelial lineage (Figure 2). Therefore, it appears that TNF-alpha is induced in the BM microenvironment and promotes BMEC apoptosis following irradiation. Whether TNF-alpha is induced and plays a role in BM carcinogenesis is not known and is the recent subject of *in vivo* studies we are currently performing. In this regard, we have preliminary evidence that TNF-alpha deficient mice may be partially protected from the leukaemia-inducing effects of irradiation. The mechanisms whereby the absence of TNF-alpha may exert a protective effect against a leukaemia carcinogenic stimulus are not known and are currently being investigated in the laboratory, although the background hypothesis is that decreased turnover of the endothelium within the BM microenvironment may have protective effects.

BM ENDOTHELIUM IN PRE-LEUKEMIA AND DURING LEUKAEMIA ONSET

Bearing in mind the importance of BMEC in leukemic disease progression (angiogenesis) we have recently focused our attention in the group of diseases termed myelodisplastic syndromes (MDS). These are common BM complications in oncology patients treated with radio- or chemotherapy. MDS are interesting diseases to study the importance of the BM microenvironment in the regulation of homeostatic BM function and also during malignant transformation, in that they represent a "pre-leukemia" stage: first there is evidence of BM apoptosis (which may be quite

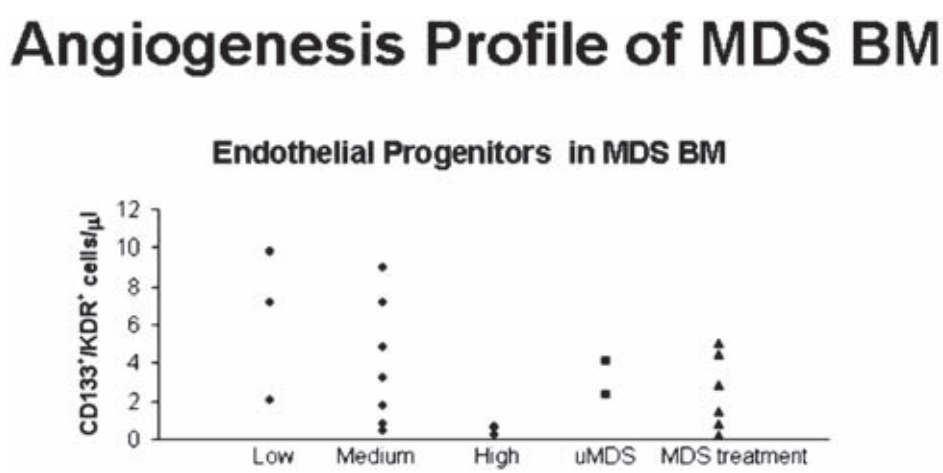

Fig. 1 – TNF-alpha levels increase in correlation with BM recovery following radiation.

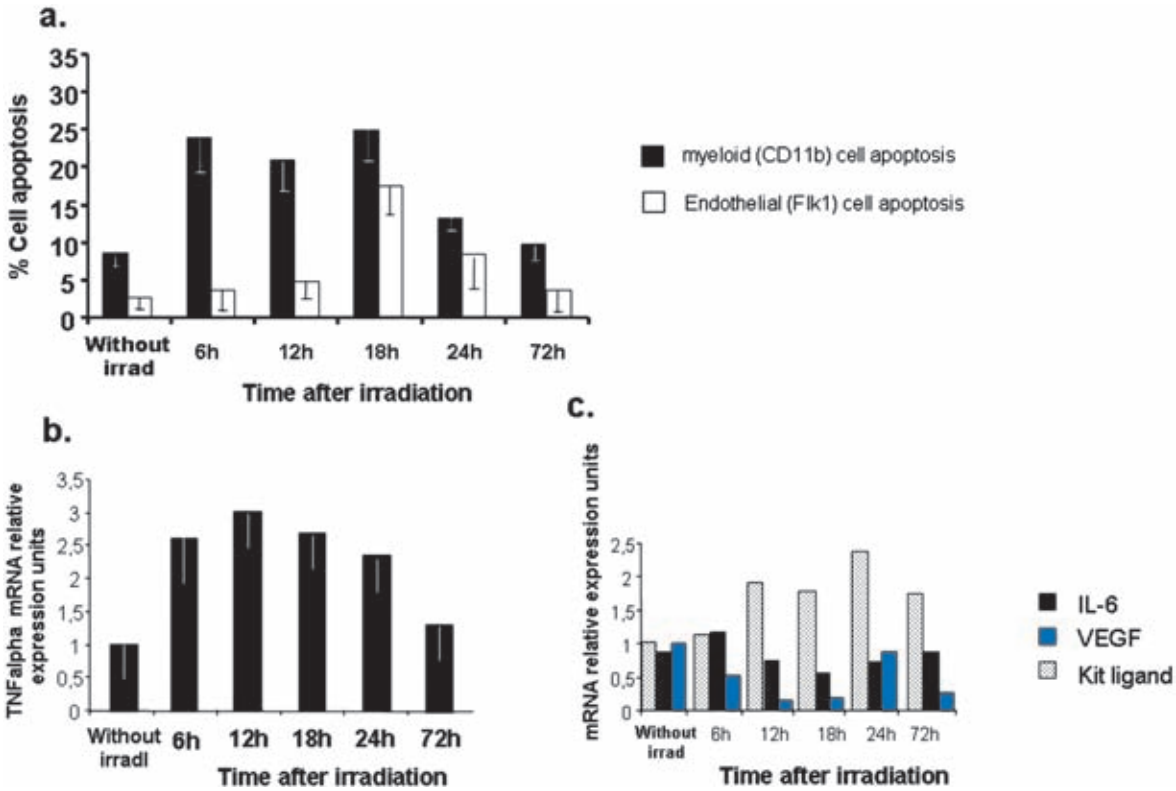

Fig. 2 – In vitro TNF-alpha blocking in total BM cell cultures decreases apoptosis incidence, most remarkably in the endothelial lineage

significant leading to cytopenias), subsequently BM turnover and selection of aggressive clones that will produce a very aggressive and un-curable acute leukemia. According to recent classifications suggested by the World Health Organization, high risk MDS have a high probability (over 80%) of progressing into acute leukaemia, while medium risk and low risk patients have reduced probabilities. Since the progression of MDS usually takes several years, these diseases provide a very interesting biological model where to study BM turnover, apoptosis and the involvement of different cell types in BM disease onset and/or progression. In our case, it was possible to isolate BM cells from patients at different stages of the disease, and in a few cases it was even possible to obtain samples from a same patient while in disease progression.

In this heterogenous group of patients, we have quantified and studied in detail the involvement of the BM vasculature (endothelial content) at the early stages of the affected BM and also during overt leukaemia progression. Interestingly, the BM endothelial progenitor (and mature endothelium) content is increased in low and medium-risk MDS patients, while the BM microvascular densities increases during the progression to leukaemia, accompanied by a significant increase in the levels (bone marrow and peripheral blood) of VEGF, and specifically of its VEGF 189 amino acids isoform (Figures 3-4).

The data obtained thus far suggests that MDS progression to leukaemia may involve (or be dependent on) an angiogenic outburst (angiogenic switch) within the BM. Nevertheless, we have evidence suggesting there is a "comsuption" of the BM endothelial content, namely in the number of endothelial progenitors, in early stages of these diseases, which is still

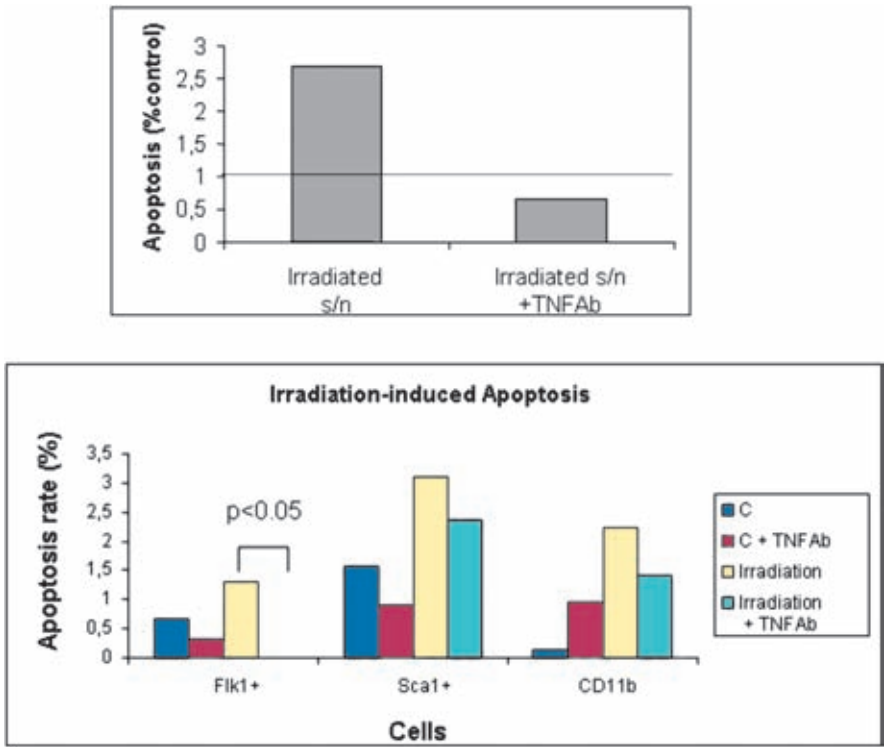

Fig. 3 – The BM endothelial progenitor (and mature endothelium) content is increased in low and medium-risk MDS patients.

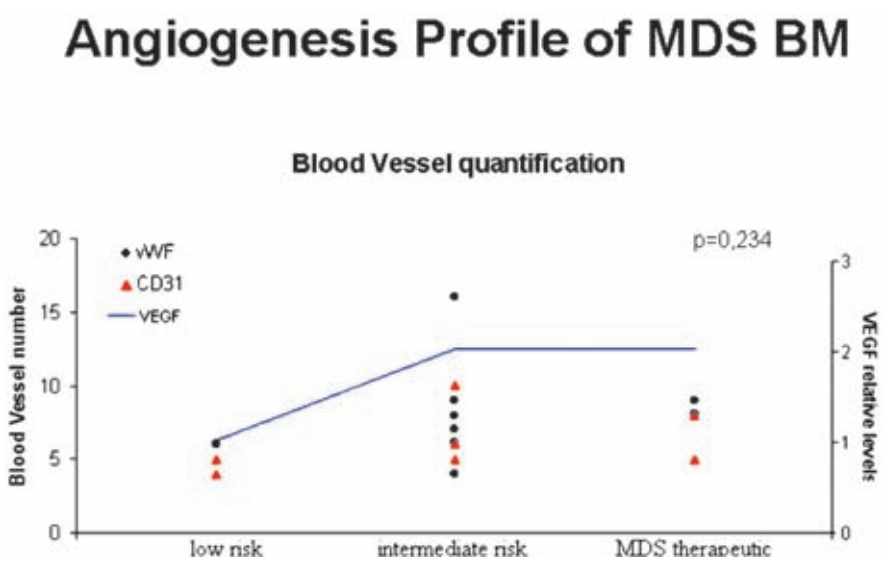

Fig. 4 – In MDS bone marrow there is an increase in vasculature with disease progression.

unexplained. Currently, we are attempting to determine the signals (hypoxia, acidosis, hypoglycaemia, etc) and the mechanisms involved in VEGF production and VEGF splicing within the BM microenvironment. In addition, recent data suggests that selective apoptosis (which may be triggered by TNF-alpha levels, for instance) of endothelial/endothelial progenitors in the BM microenvironment may facilitate leukaemia onset.

AUTOCRINE/PARACRINE STIMULATION OF LEUKAEMIA GROWTH BY VEGF

We have previously shown that acute leukemia blasts evidence an abnormal expression of VEGF receptors, whose stimulation modulates their survival, proliferation and migration. While the common expression of VEGF receptors on endothelial cells and malignant leukaemia blasts strongly suggests a putative "leukaemia-carcinogenesis hit" may be exerted at the level of a common precursor or stem cell, the fact that malignant bone marrow cells express and respond to the effects of this abundant pro-angiogenic growth factor, has been of great interest to the field of malignant hematology. In detail, we have shown that VEGF signalling on leukemia cells, via VEGFR-2 promoted their proliferation, migration while VEGFR-3 stimulation protected leukaemia cells from chemotherapy-induced apoptosis. We also demonstrated that the growth of subsets of acute leukemias is supported by both internal and external VEGF/VEGFR-2 autocrine loops, and that such loops lead to the activation of distinct signalling pathways.

More recently, we have shown acute lymphocytic leukemia expansion and the onset of extramedullary disease (EMD) involved migration of the leukemia cells, within the BM microenvironment and into the peripheral circulation. This migration was induced at least partly due to VEGF/PLGF stimulation of leukemia cells within the BM. Detailed biochemical analysis revealed that VEGF-induced leukemia migration within the BM microenvironment involved interaction between VEGFR-1, caveolae-like structures in the plasma membrane, and a close connection (activation and biochemical interaction) with the cell cytoskeleton of actin and tubulin.

Our data has revealed in detail the mechanisms whereby VEGF, produced in abundance within the bone marrow in pre-leukemia (MDS) and also during full blown leukaemia, induces transformation within the BM microenvironment suppressing selectively certain lineages, leading to the expansion of endothelial cells (and precursors) and promoting the survival (movement, proliferation and protection to apoptosis) of subsets of malignant clones.

Taken together we have been exploiting the hypothesis that sustained exposure of the bone marrow microenvironment to abnormal TNF-alpha and VEGF levels may result in selective apoptosis/turnover of endothelial cells/precursors in the BM, disruption of hematopoietic differentiation, and also promote the onset and subsequent expansion of subsets of acute leukemia. Future work includes revealing the mechanisms whereby BM endothelium may produce/establish a protective BM microenvironment to impede BM transformation in response to carcinogenesis stimuli including radiation.

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NON-NEURONAL CHOLINERGIC MECHANISMS IN RED BLOOD CELL

C. Saldanha

SUMMARY

A brief description will be done about the meaning of non-neuronal cholinergic system considering the identification of its components, the sites where they appear and the known physiological functions. The focus will be done on the red blood cell non-neuronal cholinergic responses to a stimulus.

GENERALITIES OF THE NON-NEURONAL CHOLINERGIC SYSTEM

Acetylcholine (ACh) was viewed as the "vagusstoff" after Loewi`s experiment with the frog heart. In 1936 Otto Loewi, with Henry Dale were recipients of the Nobel Prize in Physiology or Medicine, by their contribution for the acetylcholine discover as a neurotransmitter.

Outside of the central and peripheral nervous system there is evidence of the presence and of ACh that was firstly recognised in 1963 by Wittaker[1].

ACh is a phylogenetic old molecule present in prokaryotic and eukaryotic organisms. Recently Kawashima and collaborators have demonstrated the ubiquitous presence of ACh and the expression of its ACh-synthesizing enzyme among life forms without nervous system[2]. Acetylcholine participates in different cellular roles such as proliferation, differentiation, migration and immune functions, Figure 1[3-7].

ACh is considered as a universal cytotransmitter by Grando and colleagues[8,9], and is presented on epithelial, mesothelial, muscle, endothelial, and immune cells[8-10]. Endothelial cells and lymphocytes are able to synthesize and release ACh by the participation of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter respectively (VAChT)[11-13]. Lymphocytes express most of the cholinergic elements and upon interaction with antigen presenting cells or endothelial cells, T cells produces increase amount synthesis and release of acetylcholine[14]. The autocrine ACh action depending on cell membrane receptor activates or not the lymphocytic cholinergic system enhancing in case of TCR/CD3, the expression of both ChAT and M5m ACh receptors[14].

At vascular wall the intrinsic intimae non-neuronal system is presented in the endothelium,

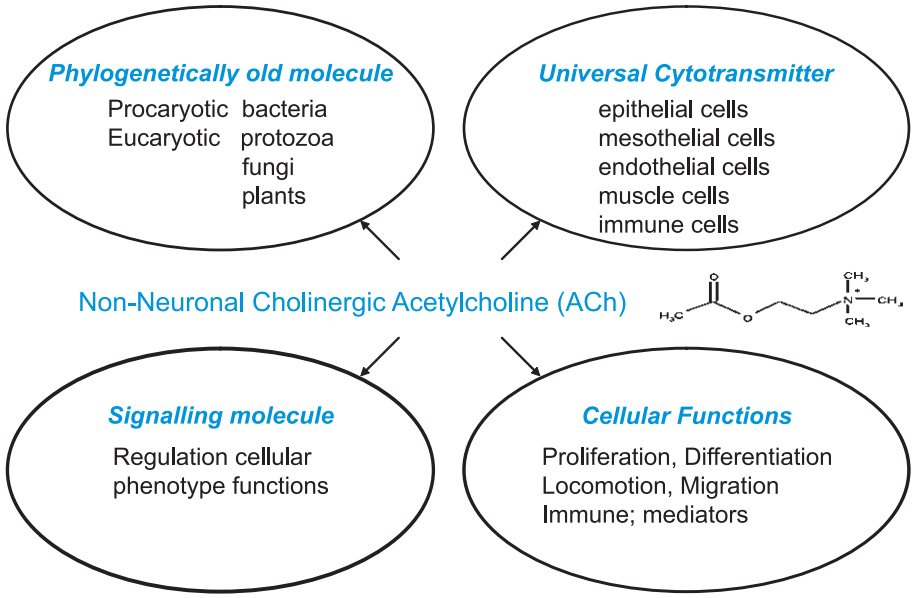

Fig. 1 – Schematic representation of the non-neuronal acetylcholine functions.

acting ACh on it by the auto-paracrine fashions[15]. Beyond the non-neuronal cholinergic components namely, ACh, ChAT and VAChT, above mentioned, the enzyme acetylcholinesterase (AChE) that conducted the hydrolysis of ACh, is also expressed[16].

We are the first to perform the biochemical characterization of human umbilical vein endothelial cell membrane (HUVECs) protein bound form of the AChE. We have identified, with C-terminal anti-AChE, the expression of one molecular form membrane with 70kDa, (the molecular mass characteristic of the human monomeric form of AChE). When the N terminal anti-AChE was used two molecular forms with approximately 66kDa and 77kDa are expressed at membrane bound level[17]. The molecular form of 70kDa is also expressed at cytoplasm and nuclear compartments, where the latter also expressed an AChE isoform with approximately 55kDA[18]. We verified that the nuclear expression is not endothelial cell-specific but is also evidenced in non-neuronal and neuronal cells.

Studies of Borovikova[19] have showed the anti-inflammatory effect of ACh in the rat's systemic inflammatory response to endotoxin. The anti-inflammatory action attribute to ACh is associated with the extrinsic vascular cholinergic system, at the perivascular nerve fibres[20].

Tracey and co-workers have recently demonstrated the existence of the cholinergic anti-inflammatory pathway, which requires the action of the parasympathetic neuronal system[19,21]. More precisely, it relies on the activity of the sensorial vagus nerve that can sense inflammatory stimuli and further provide input to brain networks (nucleus tractus solitarius in the brainstem medulla oblongata) eliciting a motor anti-inflammatory response. This "wondering" nerve innervates various organs, such as the spleen and liver. At these organs the vagus nerve terminals release ACh that further triggers a decrease in the production of pro-inflammatory cytokines by resident macrophage or other cytokine-producing cells. The "cholinergic anti-inflammatory pathway contribute with the humoral anti-inflammatory mechanisms, comprising external signals and intracellular mediators, to limited the vascular inflammation. As a result there is restraining or counter-regulating cytokine release[22].

During an inflammatory state, blood leukocyte rolls, adheres and after transmigrates through the endothelial cells where then migrates to the injured tissues[23], where there is a tissue-pool of cytokines coming from the macrophages and/or resident monocytes. The steps developed in the leukocytes/endothelial cell interactions are influenced by the tissue-pool cytokines and also from those synthesised and secreted by activated endothelial cells.

Inhibitors of AChE modulate leukocyte activation[24]. We have observed that the intravenous administration of velnacrine maleate (an inhibitor of AChE) in Wistar rats previously submitted to the administration of LPS induced an increased leukocyte adhesion to mesenteric post-capillary venules[25].

The inhibition of AChE by velnacrine also modulates leukocyte-endothelial interaction in the rat cremaster network as showed by us[26]. We observed that there is an increased number of the rolling and adherent leukocytes to the endothelial wall of the post-capillary venules of Wistar rats`mesentery muscle perfuse with velnacrine. That number of leukocytes decreased when ACh was perfused after velnacrine.

Fujii and co-workers, have determined, at normal physiological conditions, the plasma and blood levels of ACh and have verified differences among species[27].

Depending on the degree of endothelium integrity the circulating ACh induce vasodilation or vasoconstriction according the amount of nitric oxide (NO) synthesised and released[28,29].

The NO released from endothelial cells and platelets is scavenged by erythrocyte and blood cell free hemoglobin[30]. Shapiro and co-workers[31] have done NO competion experiments, between plasma cell free hemoglobin and red blood cells (RBCs), under oxygenated and deoxygenated conditions at different hematocrits. They observed that external diffusion of NO to oxygenated erythrocytes is slower than to cell free hemoglobin. The hematocrit dependence at deoxygenated conditions disappears at contrast with oxygenated conditions where lower values of erythrocyte scavenger rate in relation to cell free hemoglobin was obtained at lower hematocrit. Recently, Shapiro and collaborators[32] attribute the above results to the increased erythrocyte membrane permeability verified at anoxic conditions.

Low tissue oxygen tension induced NO participation in the hypoxic vasodilation and several studies demonstrated the involvement of the hemoglobin structural allosteric transitions as oxygen sensor[33-34]. Among the heterotrophic effectors of oxygen binding hemoglobin, NO binds to the thiol group of cysteine β93 at high tissue oxygen tension. At low tissue oxygen tension there is a NO release from either S-nitrosothiol of the S-nitrosated hemoglobin or from the reduction of the anion nitrite to NO[35,36]. It knows that the T state of SNO-Hb promotes the transnitrosation by which NO groups are transferred to thiol acceptors biomolecules in RBCs[37]. One of these is the protein band 3[38], but the exact mechanism by which NO escape from erythrocyte membrane still remain uncertain[37,38].

NON-NEURONAL CHOLINERGIC MECHANISMS IN RED BLOOD CELL

Red blood cells (RBCs) express at external membrane surface the enzyme AChE that has the particularity to be inhibited by its substrate ACh at high concentrations. Different AChE enzyme complex forms may be presented namely, active and less active ones according the amount of ACh existent.

As also referred the erythrocytes controls diffusion of small gas molecules such as NO, and inside the erythrocyte nitric oxide, can react with deoxyhemoglobin heme to form nitrosylhemoglobin or with haemoglobin thiols to form S-nitrosohaemoglobin[36,37]. For these reasons, S-nitrosohaemoglobin has been considered a reservoir of nitric oxide and as mentioned above ACh is an endog-

enous compound with vasoactive properties, present in blood circulation. We hypothesised that the non-neuronal cholinergic system participates in erythrocyte NO mobilization and translocation. So, we have questioned whether ACh induces changes in RBCs NO mobilization. In order to answer, human erythrocyte suspensions, in presence of ACh, were loading with the permeable non fluorescent probe diamino fluoresceine-2 diacetate (DAF-2Da). After we quantified by spectrofluorometry analysis the appearance of intra erythrocyte fluorescence intensity of triazolofluorescein (DAF-2T) which results from the reaction between NO and the 4, 5- diaminofluorescein, we concluded that ACh, in a concentration dependent way, is able to induce NO mobilization inside the erythrocyte[39]. Based on these results and on the vasoactive role of ACh at endothelium wall we have hypothesised that ACh induce changes in erythrocyte deformability as well as in the levels of NO metabolites namely nitrites (NO_2^-) and nitrates (NO_3^-). We have verified that in presence of ACh there is an increased of the erythrocyte deformability, of the NO_2^-, NO_3^- levels and the oxygen hemoglobin affinity and a decreased of erythrocyte aggregation, Figure 2[40,41].

The lower erythrocyte deformability expressed by hypertensive, hypercholesterolemic and kidney transplant patients was associated with a higher nitric oxide efflux under ACh stimulation as we verified by studies conducted *in vitro*[42]. This may be a compensate erythrocyte ability that allows in vivo at microcirculatory network NO and oxygen donation otherwise compromised by the erythrocyte deformability deficiency.

Human erythrocyte deformability and the oxygen hemoglobin affinity increase, without changes in membrane lipid fluidity and peroxidation, when erythrocytes were exposed to NO 10-7M. However when erythrocytes were exposed at NO 10-5M an increase in membrane lipid fluidity and peroxidation values was verified, while at NO 10-3M the methemoglobin levels increase with decreased of erythrocyte deformability[43]. The methemoglobin formation may result from the reaction between NO and oxyhemoglobin catalysed by the hemoglobin reductase enzyme where nitrates are also produced[44].

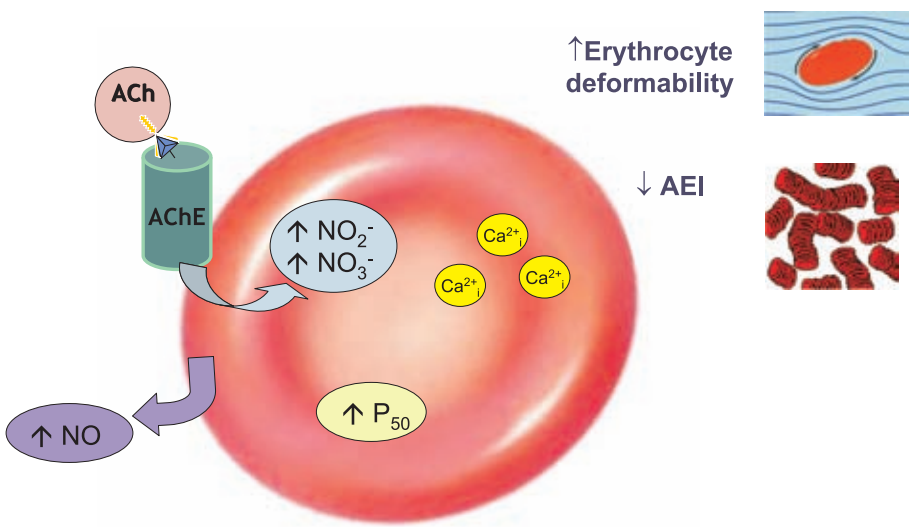

Fig. 2 – Schematic representation of the erythrocyte responses to the action of the non-neuronal acetylcholine obtained from *in vitro* studies.

If auto-oxidation of haemoglobin does occurred the superoxide anion will be produced which generates peroxynitrite after reaction with NO[45]. The decomposition of peroxynitrite molecules leads to nitrite and nitrate[46-48], and the reaction between peroxynitrite and haemoglobin generates SNOHb, which could decompose to nitrosothiol and nitrate[46-49].

Another origin for nitric oxide metabolites appearance in erythrocyte suspensions during incubation with or without ACh may resulted from the nitrosothiols decomposition (e.g. S-nitrosoglutathione) as described by Jia et al[50].

Glutathione is an abundant molecule inside erythrocytes and has a thiol group that can react with nitric oxide or other molecules to form nitrosothiols such as S-nitrosoglutathione[51]. This nitric oxide reserve attributed to glutathione could be affected by the inactivation of glutathione reductase induced by oxidative stress[52]. The thiol/disulfide reagents like as oxidised and reduced gluthathione (which is present at high level inside RBCs), has a suitable redox potencial what made it useful for regeneration proteins. For instance dithiothreitol (DTT) is a thiol reducing agent enable for regenerating disulfide-contains proteins and establish interchangeable thiol-disulfide reaction with glutathione[53]. We have hypothesised that the manipulation of erythrocyte thiol status will be able to change the NO mobilization that occurs at absence or presence of AChE effectors. We have conducted in *in vitro* studies upon redox status modulation using DTT. The following results here present are accepted for publication. We verified that NO is strongly mobilized inside RBCs but much less released to the extracellular compartment under DTT influence that when compared with the effect of acetylcholine--AChE or velnacrine-AChE complexes. Higher levels of intracellular NO are responsible for the enhanced metabolites production, explaining the greater mobilization via GSNO, although the same didn't occur with peroxynitrite concentration. DTT is an activator of the glutathione reductase enzyme activity[54] which allowing S-nitrosoglutathione renovation may be one explanation for the observed NO mobilization. Conversion of metHb into oxyHb was slightly triggered by DTT, which may be associated with a thiol-dependent activity of metHb--reductase as has been described[55].

Acetylcholine significantly prompted DTT--induced nitric oxide mobilization, since higher levels of NO and its metabolites were determined respectively in the extracellular and inner compartments. Regarding the action of velnacrine in presence of DTT, only nitrite concentration increased, while nitrate and NO values were lower. ACh or VM do not significantly modify the DTT influence on peroxynitrite levels. However, velnacrine plus DTT showed a higher increase on GSNO concentration.

An interesting finding was that reduced glutathione (GSH) concentration is not modified when DTT or AChE effectors are present, denoting that the RBCs antioxidant mechanisms are conserved. Overall peroxynitrite production was due to erythrocyte-velnacrine stimulation, while acetylcholine scarcely altered its basal levels. Reduced environment states, by turn, favoured the effects of VM but went against those of ACh in the way of higher formation of peroxynitrite.

We verified for the first time, as far as we are concerned, that the presence of increasing amounts of, DTT, do not significantly modify the red blood cell elongation index, aggregation index and membrane lipid fluidity, when incubated in blood samples of healthy subjects. However the erythrocyte deformability assessment showed a significant decrease ($p<0.05$), at low shear stress, when the AChE inhibitor, velnacrine, is present with each of the following concentrations DTT 10-6M, 10-5M 5x 10-5M in blood samples aliquots. The active ACh--AChE complex does not exert major influence on erythrocyte deformability property. Both acetylcholine and velnacrine diminish significantly the erythrocyte aggregation index, in stasis during 5 and 10 seconds, for all DTT 10-6M, 10-5M 5x 10-5M concentrations in relation to the values obtained in DTT blood samples aliquots, (CHM in

publication). These results seems, as previous documented for ACh, that the erythrocyte hemorheological profile may be dependent on its elements of the non-neuronal cholinergic system[40,42], to be in accordance with preview more general idea postulated by Paulischke and cow-workers that external and integral membrane proteins[56] influence the erythrocyte hemorheological properties.

Based in all the above mentioned reactions we have imagined a hypothesis to explain the signal transduction mechanism that may be associated with nitrite, nitrate production, and NO mobilization in presence of AChE substrate or inhibitor, in, *in vitro,* erythrocyte suspensions, Figure 3. Erythrocyte membrane protein band 3 binds some glycolytic enzymes and haemoglobin[57-59] and is the most abundant protein expressed in human red blood cell membranes. Protein band 3, known as a spanning erythrocyte membrane protein, could be phosphorylated in a tyrosine residue by protein tyrosine kinase (PTK) and then dephosphorylated by the protein tyrosine phosphatase (PTP)[60]. Besides the enrichment of shed vesicles in AChE at variance with poor band 3 protein molecules[61], we can hypothesize that changes in band 3 protein conformations could occur when ACh or velnacrine binds to AChE. In our idea either the enzyme–substrate or the enzyme–inhibitor complex induces conformational changes in an erythrocyte G protein[62], that in turn may activates the PTK enzyme responsible for band 3 phosphorylation with sorting of glycolytic enzyme and consequently increasing the glycolytic pathway rate. The NADH produced may participate in the methahemoglobin conversion to haemoglobin by haemoglobin reductase action[63].

The S-nitrosohaemoglobin (SNOHb) molecules binding to the phosphorylated band 3 protein, that has exposed SH group, allows the trans-nitrosylation reaction with the band 3. The NO·molecules after be transferred are released from the erythrocytes[64].

In summary, we hypothesis a possible acetylcholinesterase role in the signal transduction mechanism in response to the action of acetylcholine that originate NO mobilization and nitrite and nitrate changes concentrations, in human erythrocyte suspensions. The trans-nitrosylation process coupled between band 3 proteins and SNOHb could be associated with an unknown mechanism mediated by the AChE-ACh complex, with the participation of PTK and PTP which may be dependent on G protein (G Prot)[41].

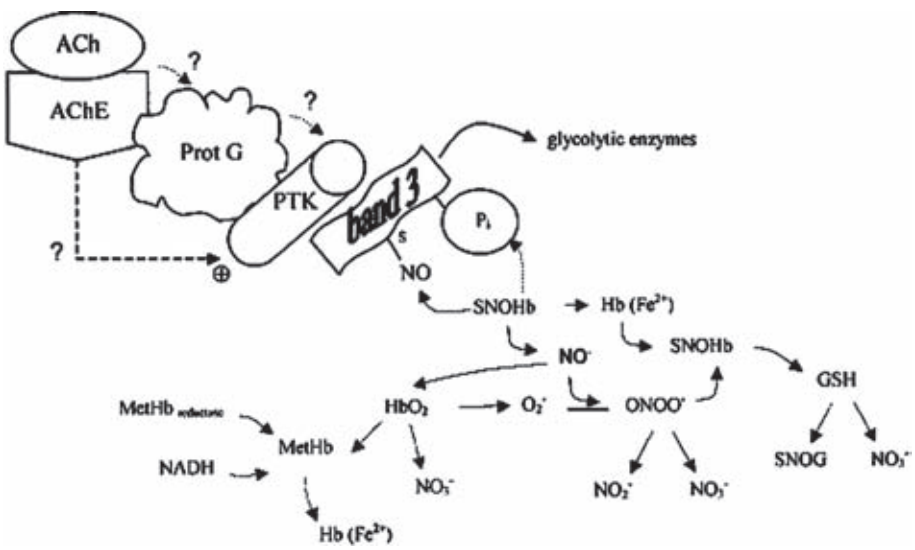

Fig. 3 – Schematic representation of the signal transduction mechanism proposed for the erythrocyte non-neuronal cholinergic system[41].

In order to verified our hypothesis we start by studying the influence of the band 3 protein phosphorylation degree in the RBCs hemorhreological properties. After we have studied the influence of the AChE substrate and inhibitor on the band 3 phosphorylation degrees, and we have identified the G protein associated with these band 3 states of phosphorylation in absence and presence of AChE effectors.

When we modulate protein band 3 phosphorylation states with PTK $p53/56^{lyn}$ inhibitor (Aminogenistein or AMGT) and PTP inhibitor (Calpeptin), the erythrocyte elongation index (EEI), or deformability, was not affected[65]. At variance erythrocyte aggregation increased when band 3 protein is phosphorylated and decreased when at dephosphorylated state. However both manipulated states induced lower erythrocyte aggregation values than blood samples aliquots control[65].

Concerning the AChE effectors ability to modify erythrocyte NO translocation we recall what is mentioned above, that manipulated red blood cells suspensions in the presence of ACh, shows increase of NO levels translocation. However the addition of $p72^{syk}$ inhibitor, (band 3 protein at partially phosphorylated state), to the RBCs suspensions, reveal a decrease of NO concentrations besides the presence of ACh. When in presence of calpeptin and ACh, band 3 being totally phosphorylated, we observed an increase of NO levels. The erythrocyte NO mobilization and its efflux are dependent of the AChE-ACh enzyme active complex and also from the protein band 3 phosphorylation degree, (in publication).

The addition of PTK_i, (inducing a band 3 protein partially phosphorylated state), showed higher NO_x levels in the presence of the velnacrine--AChE less active enzyme complex. In the presence of calpeptin, (protein tyrosine phosphatase inhibitor), band 3 being totally phosphorylated, we obtained the opposite NO mobilization.

Triton X-100 erythrocyte membrane soluble extracts revealed that the level of phosphorylated band 3 obtained in control samples was influenced by the presence of acetylcholine, velnacrine, calpeptin, and PTK_i. ACh maintains the level of band 3 phosphorylation induced by calpeptin at variance with the antagonism action caused by the less active enzyme AChE –velnacrine form. Both active and less active enzyme AChE complex do not abolished the dephosphorylation state of band 3 induced by the protein tyrosine kinase inhibitor.

These results suggested the key-role of the AChE-effectors enzyme complexes in the band 3 phosphorylation/dephosphorylation reactions PTK and PTP dependent in association with erythrocyte NO metabolism.

Recalling our hypothesis for the signal transduction mechanism the involvement of a G protein needs to be confirmed as well as its type.

From the literature it was described that the heterotrimeric G protein Gi, participates in the ATP release from erythrocytes[66] that the expression of G_{i2} is reduced in the erythrocyte membranes of humans with type 2 diabetes[67]. It was also verified a decreased of erythrocyte G proteins (Gαi, Gαo and Gβ) in hypertensive subjects in relation to healthy persons[68].

Our purpose was to identify the G protein type that could be linked to the band 3 protein phosphorylation degree states and to know whether each G protein sub-units (α,β,γ) is related to the active or less active enzyme AChE complex forms. For this propose we made *Western blotting* analysis using primary antibodies to different protein G sub-units such as anti-protein G_{β}, anti-protein $G\alpha_{i1/2}$, anti-protein $G\alpha_{i3}$, anti-protein $G\alpha_{i3}/G\alpha_{0}$, anti protein $G\alpha_{S}$ and anti-protein $G\alpha_{q/11}$. We could identify on erythrocytes membrane soluble extracts possible linkage between protein $G\alpha_{i1/2}$ and/or protein G_{β} with protein band 3. The results were then confirmed by immunoprecipitation of this two protein G sub-units with following analysis by Western blot using antibodies against protein band 3 (C-terminal) and band 3 (N-terminal).

From all the blood samples aliquots studied we verified that G protein sub-units $G\alpha_{i1/2}$ and G_{β} is linked with band 3 C-terminal site. Moreover when erythrocyte AChE was stimulated with acetylcholine and when is present with PTK inhibitors there was

an increase of the expression of the linkage between $G\alpha_{i1/2}$ – Band 3 (C- and N-terminal) and G_{β} – Band 3 (C –terminal). These two conformational states of G protein sub-units seem to be related with the phosphorylation band 3 protein states.

Our results of this work allow us to identify the linkage between protein Gαi1/Gαi2 and/or protein Gβ and protein band 3 on erythrocytes membrane. At band 3 C-terminal end both protein Gαi1/Gαi2 and protein Gβ are bonded. We observed that when the erythrocyte active AChE/ACh enzyme complex is formed in absence or presence of PTK inhibitors, there is an increase of the linkage Gαi1/Gαi2 – band 3 protein at C-terminal domain.

In summary, our data allows us to confirm that G_i protein is a necessary component of the signal transduction pathway that seems linked to the band 3 protein phosphorylation degree states and related to the activation or inhibition of acetylcholinesterase complex. We can conclude that our proposed signal transduction mechanism based on NNCS participates on erythrocyte NO translocation and contribute to understand some intracellular erythrocyte-dependent events. Some insights may contribute to better understand the erythrocyte functions at microcirculation level at physiological and pathological conditions

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ANGIOGENESIS IN BREAST CARCINOMAS WITH DIFFERENT EXPRESSION PROFILES

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ABSTRACT

Endoglin is a glycoprotein preferentially expressed in proliferating vessels during angiogenesis. It is described that high angiogenic index detected by endoglin is associated with poor prognosis in breast cancer patients. This indicates that endoglin could serve as a target for antiangiogenic therapy. However, the relationship between angiogenic index and the molecular subtypes of breast carcinoma is not yet extensively explored, especially in the basal-like subset, in which the anti-angiogenic therapy could be an option, since these carcinomas still do not have a specific therapy.

We have studied the expression of endoglin on formalin-fixed paraffin-embedded tissue sections of invasive breast carcinomas by using immunohistochemical assays in a series of 161 patients.

Our results show that although the angiogenic index is higher in the basal-like subtype than in the other groups of breast carcinomas, this difference is not statistically significant. This suggests that the use of an antiangiogenic therapy can be valid in all the subtypes of breast cancer, as combined therapy with chemotherapeutic agents.

INTRODUCTION

Angiogenesis is a complex multistep process required for tumour growth and metastasis. It involves endothelial cell migration and proliferation, microvessel differentiation and anastomosis, and extracellular matrix remodelling[2].

Studies have shown that endoglin (CD105) is involved in the development of blood vessels and that it represents a specific marker of neovascularization for several types of tumours[4]. Endoglin is a cell-surface glycoprotein recently identified as an optimal indicator of human endothelial cells proliferation. Furthermore, the angiogenic index detected by its expression has been correlated with poor prognosis in breast cancer patients[3]. Collectively, these data suggest that the tumour microvasculature may constitute a relevant target for antiangiogenic therapy. Specifically, endoglin, because of its preferential expression in the newly formed endothelium, can be considered a potential target for this kind of treatment. Despite these evidences, the relationship between angiogenesis, assessed by the immunohistochemical expression of endoglin, and the molecular subtypes of breast carcinomas has not yet been addressed.

Gene expression profiling has classified invasive breast carcinomas into different subtypes[6], based on the expression of two molecular markers: ER and HER2. Thus, tumours can be classified as: Luminal A type (ER+/HER2-), Luminal B (ER+/HER2+), basallike (ER-/HER2-) or HER2-overexpressing carcinomas (ER-/HER2+). Basal-like breast carcinomas are also characterised by expression of basal/myoepithelial cell markers, such as CK5, P-cadherin, EGFR and p63 (5). Unlike estrogen receptor positive cancers, that respond well to hormonal therapy and HER2-overexpressing tumours that are responsive to Trastuzumab, basal-like carcinomas do not have a specific therapy and display a preferential hematogenic pattern of metastasis.

The aim of the present study was to evaluate the relationship between angiogenesis, assessed by the immunohistochemical detection of endoglin and the molecular subtypes of human invasive breast carcinomas, defined by microarray gene expression profiling and validated by immunophenotype. Also, we would like to clarify if basal-like carcinomas have a higher angiogenic index and whether there is evidence to indicate that endoglin could be a potential target for antiangiogenic therapy, especially in the basal-like subset of breast carcinomas.

METHODOLOGY

We have studied a cohort of 161 cases of invasive breast carcinomas, collected from the archives of the Pathology Department of the Federal University of Santa Catarina, Florianópilis, Santa Catarina, Brazil.

Immunohistochemical staining for endoglin (CD105) in two-micron thick sections of whole tissue was performed using the streptavidin-biotin-peroxidase method. Antigen unmasking was carried out using a commercially available solution of citrate buffer pH=6,0 (Vector Laboratories, Burlingame, CA, USA) at 98ºC for 30 minutes. The slides were incubated with the primary antibody anti-CD105, clone 4G11 (Novocastra, UK) in a 1:50 dilution overnight at 4ºC and a DAB solution (3,3-diaminobenzidinetetrahydrochloride) (DakoCytomation, Carpinteria, CA, USA) was used as a chromogen. Paraffin sections of a breast invasive carcinoma were used as positive controls in every run.

The samples had been previously tested for ER, HER2, CK5, EGFR, P-cadherin and p63 status in Tissue Microarrays.

We used the Microvessel Density to assess the expression of endoglin. For that, we chose 3 hot spots areas in each sample and counted the stained blood vessels in the observational field of 200x magnification, as previously described[1]. Then, we calculated the mean value of stained vessels per case. The X^2 contingency test was used for cate-

gorical variables to determine associations between groups (the various subtypes of breast tumours and the expression of endoglin). A p value < 0,05 was considered to represent a significant difference.

RESULTS, DISCUSSION AND CONCLUSIONS

Endoglin was expressed in the vascular endothelial cells in almost all cases. The staining was observed in the endothelial cell membrane and cytoplasm of newly-formed vessels (Figure 1).

Then, we grouped the cases according to their immunohistochemical profile for ER, HER2 and basal markers and calculated the mean value of stained vessels per tumour subtype.

We demonstrated that the angiogenic index measured by endoglin was similar in all subtypes of breast carcinomas (Table I and Figure 2).

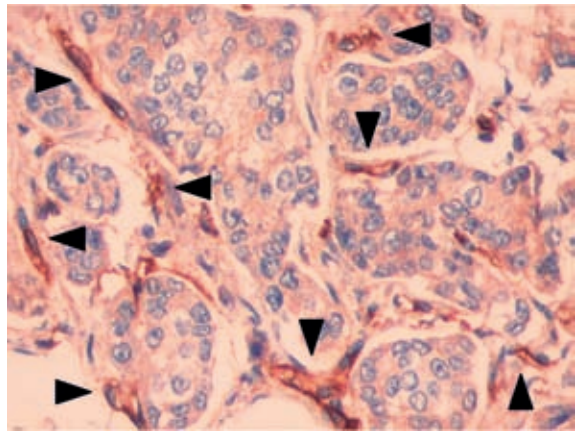

Figure 1 – Endoglin immunohistochemical staining of newly-formed blood vessels (arrows) in an invasive breast carcinoma (magnification of 400X)

The number of stained vessels was higher in the basal-like subgroup (the mean value is 28,8 microvessels per mm^2), in comparison with the other molecular subtypes (27,3 microvessels per

Table I – Expression of endoglin in the distinct breast tumour molecular subtypes

Tumour subtype	Stained vessels (mean values per mm^2)	Standard deviation
Luminal A	24,9	11,4
Luminal B	18,6	3,8
Basal-like	28,8	9,7
HER2-overexpressing	27,3	10,3

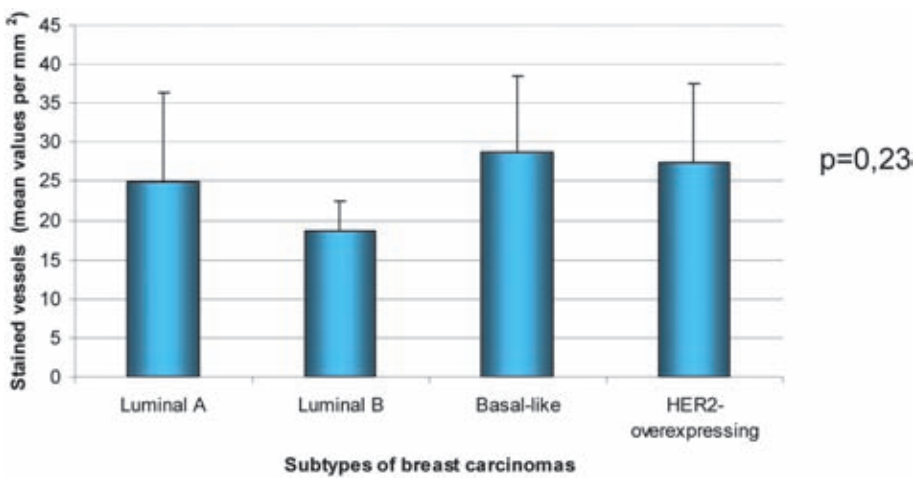

Figure 2 – Expression of endoglin in the various breast tumour molecular subtypes

mm^2 in HER2 overexpressing carcinomas, 24,9 microvessels per mm^2 and 18,6 microvessels per mm^2 for luminal A and luminal B, respectively). However, these differences are not statistically significant (p=0,23). This suggests that endoglin is not a good discriminator of the different subtypes of breast cancer.

Although these results should be validated in different and larger series, they show that basal-like breast carcinomas have a similar angiogenic index when compared to the other subtypes, and that an antiangiogenic therapy can not be claimed as a specific therapy to this subset of breast carcinomas, which does not avoid its use in combination with chemotherapy for all the subsets of breast carcinomas.

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ERYTHROCYTE DAMAGE/AGING/REMOVAL ARE ENHANCED IN BOTH MOTHER AND FETUS, IN PREECLAMPSIA

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SUMMARY

The development of preeclampsia (PE) is linked to a failure of trophoblastic invasion of the spiral arteries. Within these arteries, the deposition of fibrinoid material and foam cells in PE may lead to a reduced blood flow, favouring the interaction between the surrounding cells. The longer exposure of red blood cell (RBC) to oxygen metabolites and proteases produced by inflammatory cells may account for RBC damage. We aimed to study if a continuous enhanced exposure to inflammatory activation products throughout a preeclamptic (PEc) gestation, would account for a higher RBC damage, which may compromise oxygen maternal-fetal exchange and, therefore, placental homeostasis and fetus development.

The study was performed in 42 healthy pregnant women and 44 preeclamptic pregnant women, and in their neonates. We evaluated maternal erythrocyte changes [RBC count, hemoglobin (Hb), hematocrit (Ht), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), reticulocyte and nucleated RBC (NRBC) count, reticulocyte production index (RPI)] occurring in normal and PEc pregnancies and their relationship with the erythrocyte changes of their neonates. As markers of oxidative and proteolytic stress, membrane bound haemoglobin (MBH) and the profile of erythrocyte membrane protein band 3 [% of monomers, High molecular weight aggregates (HMWAg) and proteolytic fragments] was evaluated, as well as bilirubin concentration, as a marker of hemoglobin turnover. RBCs under oxidative and/or proteolytic stress are known to be marked for death by a rise in MBH and band 3 modifications.

PEc mothers presented significantly higher values for MBH, HMWAg, RBC count, Hb, Ht, reticulocyte count, RPI and bilirubin concentration; no morphological RBC changes were observed.

When comparing newborns from normal and PEc mothers, we observed similar values for HMWAg, RBC count, Hb and Ht, though significantly higher MBH, MCH, MCV, reticulocyte, RPI and NRBC values were observed, and a trend to higher values of bilirubin concentration.

Our data suggest that maternal blood changes and the abnormal remodelling of placental spiral arteries in PE, seem account for a higher RBC damage/aging/removal in both mother and fetus and may somehow compromise the placenta transfer mechanisms and fetal growth.

INTRODUCTION

Normal pregnancy leads to an inflammatory process, which seems to be enhanced in preeclampsia (PE). The development of PE is linked to a failure of trophoblastic invasion of the spiral arteries. Within these arteries, the accumulation of fibrinoid material and "foam" cells may lead to a reduced blood flow, favouring the interaction between the surrounding cells. A longer exposure of red blood cells (RBC) to oxygen metabolites and proteases produced by inflammatory cells may account for RBC damage.

We aimed to study if a continuous enhanced exposure to inflammatory activation products throughout a preeclamptic (PEc) gestation, would account for a higher RBC damage, which may compromise oxygen maternal-fetal exchange and, therefore, placental homeostasis and fetus development. We evaluated maternal erythrocyte changes [RBC count, hemoglobin (Hb), hematocrit (Ht), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), reticulocyte and nucleated RBC (NRBC) count, reticulocyte production index (RPI)] occurring in normal and PEc pregnancies and their relationship with the erythrocyte changes of their neonates. As markers of oxidative and proteolytic stress, RBC membrane bound hemoglobin (MBH) and the profile of erythrocyte membrane protein band 3 [% of monomers, high molecular weight aggregates (HMWAg) and proteolytic fragments] were evaluated, as well as bilirubin concentration, as a marker of hemoglobin turnover. RBCs under oxidative and/or proteolytic stress are known to be marked for death by a rise in MBH and band 3 modifications.

MATERIALS AND METHODS

Approval for the study was given by the Ethics Committee of the Hospital S. João, Porto. PE was defined according to established criteria as a systolic/diastolic blood pressure of at least 140/90 mmHg (after 20 weeks gestation) and proteinuria of at least 1+ (30mg/dl) on dipstick testing, both on 2 occasions, 4 to 6 hours apart[1].

The study was performed in 42 healthy pregnant women and 44 PEc pregnant women, and in their neonates. Blood was collected from pregnant women before delivery, and UCB was obtained after delivery of the placenta.

Hematologic study

RBC count, Hb, Ht, MCV, MCH, MCHC were measured by an automatic blood cell counter (Abx Micros 60). NRBC count was evaluated in Wright stained blood films. Blood films using supravital new methylene blue stain (reticulocyte stain; Sigma, St Louis, MO, USA) were prepared to evaluate reticulocyte count.

Bilirubin

Total bilirubin was quantified by a colorimetric method (Bilirubin, Randox).

Erythrocyte damage

Band 3 profile:

RBC were isolated by centrifugation in a density gradient. Washed RBC were lysed and washed according to Dodge method[2] and using PMSF (as a protease inhibitor) in the first two washes. Protein concentration of the obtained membrane suspensions was evaluated by the Bradford method[3].

After SDS-PAGE a RBC membrane proteins and electrophoretic transfer to nitrocelulose sheet, the immunoblot for band 3 was performed[4] and band 3 profile quantified by densitometry (*Bio1D++ version 99*, Vilber Lourmat, France).

Membrane bound hemoglobin (MBH):

Measured spectrophotometrically at 415 and 700 nm, expressed in function of protein content[4].

STATISTICS

Data normally distributed are presented as mean ± standard deviation and compared using the Student *t* test. Data not normally distributed are presented as medians and interquartile range (IQR) and compared using Mann-Whitney U test and Wilcoxon Signed Ranks Test. Correlations between variables were evaluated by the Spearman´s correlation coefficient (*r*). Significance was accepted at *P* less than 0.05.

RESULTS

Clinical characteristics of the studied groups are presented in Table I.

PEc pregnancy, when compared with normal pregnancy, presented significantly higher blood pressure; similar maternal age and body mass index (BMI) and significantly lower gestational age and newborn weight.

Figure 1 presents the immunoblots for band 3 of two studied cases (a normal and a PEc case).

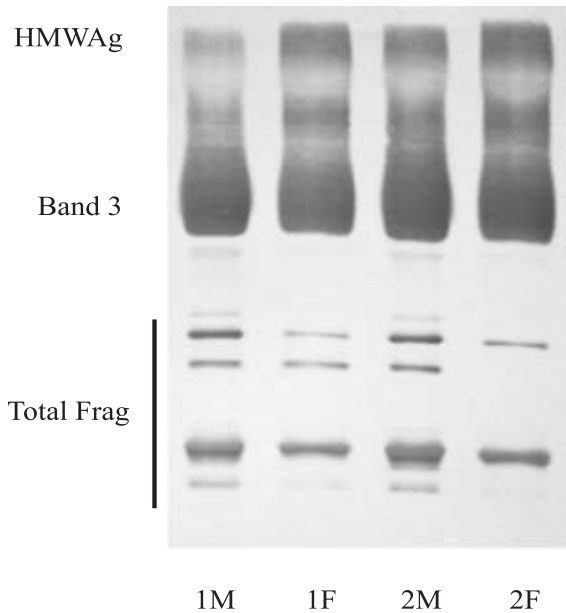

Figure 1 – Band 3 immunoblots of two studied cases. 1M: normal pregnant woman; 1F: UCB from normal pregnancy; 2M: PEc pregnant woman; 2F: UCB from PEc pregnancy.

Table I – Clinical data of normal and preeclamptic groups at delivery [mean ± SD or median (IQR)].

	Normal	Preeclamptic	P
Maternal characteristics:			
Gestational Age (wk)	38.5 (38.0; 39.3)	37.0 (34.3;38.0)	< 0.001
BMI (kg/m^2)	29.2 (27.2; 30.8)	29.8 (26.8; 33.0)	0.15
Age (y)	30.4 ± 5.7	29.7 ± 5.2	0.61
Blood Pressure (mm Hg):			
Systolic	119.9 ± 11.5	155.0 ± 14.9	< 0.001
Diastolic	69.0 ± 7.2	97.4 ± 6.3	< 0.001
Fetal characteristics:			
Birth weight (kg)	3.4 (3.0; 3.7)	2.6 (1.9; 3.1)	< 0.001
SGA (n)	0 (0%)	6 (13.6%)	< 0.01

BMI – Body Mass Index; **SGA** – Small for Gestational Age

The Table II presented the hematologic study, bilirubin levels and the band 3 profiles for the studied groups (percentage of band 3 monomer, high molecular weight aggregates; total proteolytic fragments).

Comparing normal with PEc pregnant women, we observed significantly higher RBC, Hb, Ht, reticulocyte count, RPI, bilirubin, HMWAg and MBH for PEc group.

We found that MCV, MCH, NRBC, reticulocyte count, RPI and MBH were significantly higher in UCB from PEc pregnancy, when compared with normal pregnancy.

All parameters, except MCHC and Total Frag, were significantly higher in UCB (normal and PEc) than in maternal blood.

We also found significant positive correlations between maternal and cord blood for MBH (in normal and PEc pregnancy, Fig. 2), for HMWAg (in normal and PEc pregnancy, Fig. 3) and for Bilirubin (in PEc pregnancy, Fig. 4). We also found a significant positive correlation between

Table II – Hematologic study and bilirubin levels of normal and preeclamptic groups [median (IQR)].

	Maternal		P Value	Fetal		P Value
	Normal (n =42)	PEc (n =44)		Normal (n =40)	PEc (n =44)	
RBC ($x10^{12}$/l)	3.7 (3.5; 4.0)	4.2 (3.9; 4.4)	<0.001	4.4 (4.1; 4.6)	4.3 (4.0; 4.7)	0.38
Hb (g/dl)	11.5 (10.9; 12.4)	12.7 (11.8; 13.8)	<0.001	15.5 (14.8; 16.2)	16.0 (14.7; 17.2)	0.12
Ht (%)	34.0 (32.0; 36.1)	38.5 (35.1; 40.8)	<0.001	45.9 (43.2; 49.6)	47.2 (44.5; 50.8)	0.28
MCV (fl)	91.0 (87.8; 94.0)	91.6 (89.1; 94.4)	0.18	104.5 (103.0; 107.8)	110.0 (106.9; 114.6)	<0.001
MCH (pg)	30.6 (29.8; 31.9)	30.8 (29.8; 31.9)	0.73	35.5 (34.3; 36.2)	36.5 (35.6; 38.5)	<0.001
MCHC (g/dl)	33.9 (33.1; 34.6)	33.5 (32.9; 34.2)	0.15	33.6 (33.0; 34.2)	33.7 (32.9; 34.3)	0.51
Reticulocytes ($x10^{9}$/l)	36.0 (22.8; 57.8)	71.4 (47.3; 96.3)	<0.001	154.0 (96.6;191.2)	158.0 (138.1;205.3)	0.048
RPI	0.60 (0.30; 0.84)	1.40 (0.90;1.90)	<0.001	3.40 (2.20; 4.60)	4.00 (3.20; 5.20)	0.020
NRBC ($x10^{9}$/l)				0.39 (0.16; 0.90)	0.59 (0.28; 1.69)	0.016
Bilirubin (mg/dl)	0.50 (0.40; 0.70)	0.80 (0.60; 0.80)	0.001	1.30 (1.13; 1.56)	1.50 (1.13;1.78)	0.086
MBH (%$x10^{-4}$)	73.0 (66.3; 95.8)	86.0 (70.5; 126.0)	0.016	228.8 (188.3; 294.3)	331.0 (245.5; 420.3)	<0.001
HMWAg (%)	14.9 (10.7; 18.3)	16.3 (13.7; 18.9)	0.040	19.9 (17.7; 22.8)	19.5 (18.5; 21.8)	0.61
Band 3 (%)	57.1 (51.9; 62.0)	54.0 (49.0; 60.9)	0.17	60.7 (56.0; 65.4)	61.2 (55.4; 65.5)	0.77
Total Frag (%)	28.0 (21.9; 35.3)	29.4 (20.9; 34.9)	0.80	17.3 (14.1; 25.8)	18.0 (14.4; 25.3)	1.00

Total frag, includes proteolytic fragments (60, 40 and 20 kDa); PEc, Preeclamptic
(a) Mother/Fetus in normal pregnancy; (b) Mother/Fetus in PEc pregnancy

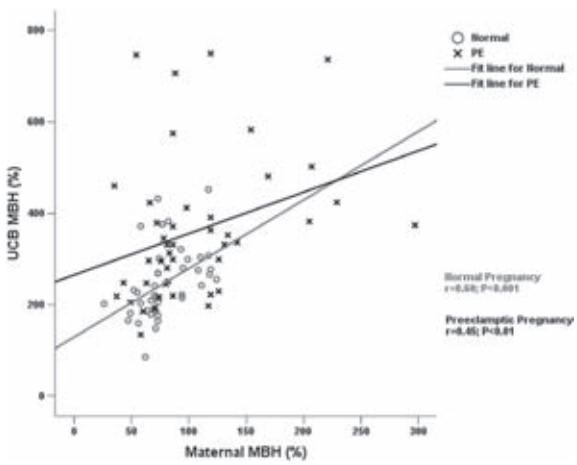

Figure 2 – Maternal vs cord blood MB

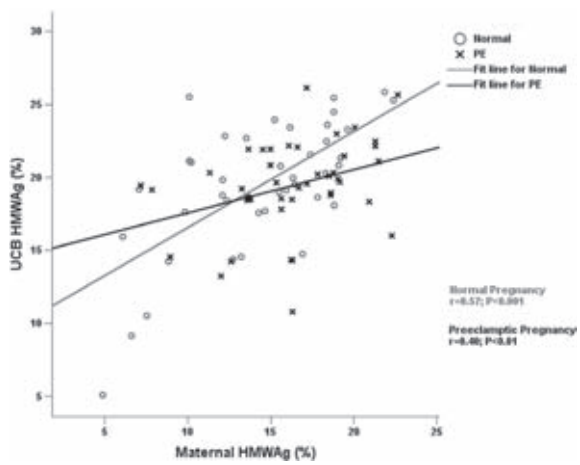

Figure 3 – Maternal vs cord blood HMWAg

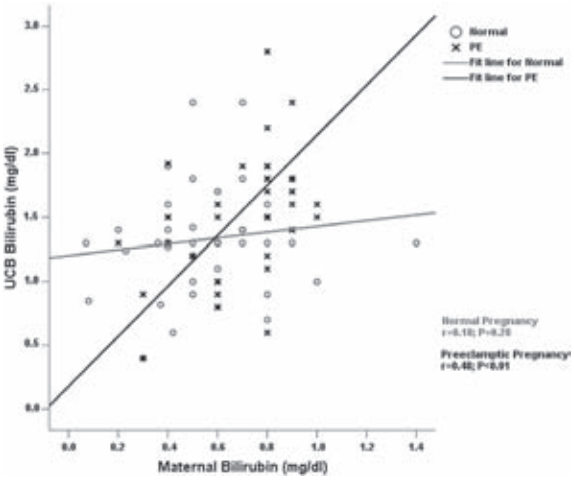

Figure 4 – Maternal vs cord blood bilirubin

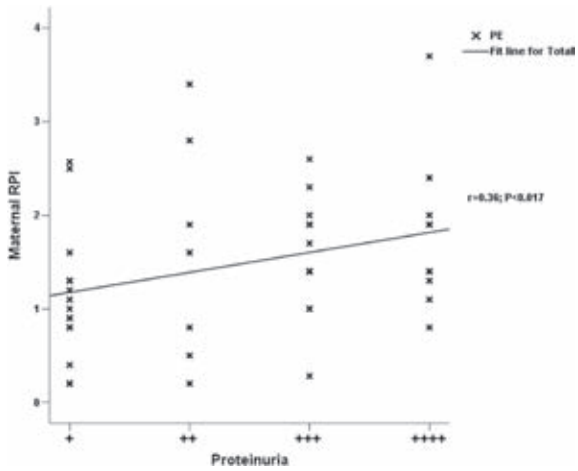

Figure 5 – Proteinuria vs Maternal RPI

maternal RPI and Proteinuria (marker of PE severity) (Fig. 5).

CONCLUSIONS

Our data show a higher RBC damage in both mother and fetus in PE that might be linked to maternal blood changes and to hemorheological disturbances due to the abnormal remodelling of placental spiral arteries[5]. Our data suggest that in PEc women a higher RBC damage/removal occurs, as suggested by the significant increase in bilirubin, in MBH and in band 3 aggregation (HMWAg), triggering a higher physiological RBC production, as reflected by the higher reticulocyte count and RPI values. This increase in RBC damage and/or removal in PEc mothers and in their newborns may be also a reflection of a placental hypoxic condition. A relationship between maternal and fetal changes seems to occur, as suggested by the observed correlations between maternal and fetal markers of RBC damage. Moreover, we propose MBH and band 3 profile as good markers of RBC damage in PE.

ACKNOWLEDGEMENTS

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ENDOTHELIAL FUNCTION IN NEWBORN INFANTS FROM PREECLAMPTIC PREGNANCIES

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SUMMARY

Preeclampsia (PE) is a characteristic hypertensive disorder of human pregnancy that is potentially dangerous for both mother and fetus. It is widely accepted that the fibrinolytic system is altered in PE, and is likely to result from the underlying endothelial dysfunction observed in this syndrome. A significant increase in PAI-1 antigen as well as in tPA antigen has been observed in PE and these may work as markers of endothelial dysfunction.

Our aim was to evaluate hemostatic variables in normal and PEc pregnancies at delivery, both in maternal and umbilical cord blood (UCB). We measured the antigen plasma levels of tissue plasminogen activator (tPA) and of plasminogen activator inhibitor type 1 (PAI-1), both markers of hemostatic and endothelial function disturbances, and fibrin fragment D-dimer.

Maternal blood from uncomplicated (n=42) and PEc pregnancies (n=44) were collected before delivery, and UCB immediately after delivery of the placenta. We found significantly higher values for PAI-1 and tPA in PEc women when compared with normal pregnant women, but no significant difference was found for D-dimer. In UCB, only tPA was significantly higher in PEc cases. In women with PE, proteinuria (marker of PE severity) correlated positively and significantly with tPA (r=0.44, P= 0.003) and PAI-1 antigen levels (r=0.58, P< 0.001). An inverse relationship between maternal tPA antigen levels and fetal birth weigh in PE (r=-0.63, P< 0.001) was also observed.

In summary, tPA and PAI-1 levels are higher in PEc women, suggesting endothelial dysfunction, and correlate with the severity of PE. Furthermore, these PEc hemostatic changes seem to have impact in fetal circulation. We suggest that tPA may be a good marker of fibrinolytic impairment and of endothelial dysfunction, particularly in the maternal circulation, and that the impact of raised tPA levels in the neonates from PEc mother deserves further studies.

INTRODUCTION

Preeclampsia (PE) is a characteristic hypertensive disorder of human pregnancy that is potentially dangerous for both mother and fetus. It is widely accepted that the fibrinolytic system is altered in PE, and is likely to result from the endothelial dysfunction observed in this syndrome[1]. A significant increase in plasminogen activator inhibitor type 1 (PAI-1) antigen as well as in tissue plasminogen activator (tPA) antigen has been observed in PEc pregnant women and these may work as markers of endothelial dysfunction.

Our aim was to evaluate hemostatic variables in normal and PEc pregnancies at delivery, both in maternal and umbilical cord blood (UCB). We measured the antigen plasma levels of tPA and of PAI-1, both markers of hemostatic and endothelial function, and fibrin fragment D-dimer.

MATERIALS AND METHODS

Approval for the study was given by the Ethics Committee of the Hospital S. João, Porto. PE was defined according to established criteria as a systolic/diastolic blood pressure of at least 140/90 mmHg (after 20 weeks gestation) and proteinuria of at least 1+ (30mg/dl) on dipstick testing, both on 2 occasions, 4 to 6 hours apart[2].

Blood was collected from normal (*n= 42*) and PEc pregnant women (*n= 44*) before delivery and UCB was obtained after delivery of the placenta.

PAI-1 and tPA antigen and D-dimer levels were evaluated by using enzyme-linked immunoadsorbent assays (Biopool).

STATISTICS

Data analysis was performed using Statistical Package for Social Sciences (SPSS) version 13.0 for Windows (SPSS Inc, Chicago). Kolmogorov-Smirnov analyses were used to test if the results were normally distributed. Clinical data are presented as mean ± standard deviation (data normally distributed) or median (interquartile range). Comparisons between groups were made by Student's unpaired *t* test. Data not normally distributed were compared by the Mann-Whitney *U* test and the Wilcoxon Signed Ranks test. The proportion of small for gestational age (SGA) cases was compared between normal and PEc pregnancies by Chi-square test. The strength of the association between the substances was estimated by Spearman's rank correlation coefficient. *P*-values below 0.05 were considered statistically significant.

RESULTS

Clinical characteristics of the studied groups are presented in Table I.

PEc pregnancy, when compared with normal pregnancy, presented significantly higher blood pressure; similar maternal age and body mass index (BMI) and significantly lower gestational age and newborn weight.

Comparing normal with PEc pregnant women, we observed significantly higher PAI-1 and tPA levels for PEc group.

We found that tPA was also significantly higher in UCB from PEc pregnancy, when compared with normal pregnancy. TPA levels were significantly higher in pregnant women (normal and PEc) than in UCB.

We also observed that in the PEc group, proteinuria (a marker of PE severity) correlated positively with maternal PAI-1 levels (Fig 1) and maternal tPA levels (Fig 2). We also found a significant positive correlation between maternal tPA and maternal D-dimer in normal pregnancy (Fig 3) and a significant inverse relationship between maternal tPA antigen levels and fetal birth weight in PE (Fig 4).

Table I – Clinical data of normal and preeclamptic groups at delivery [mean ± SD or median (IQR)].

	Normal	Preeclamptic	P
Maternal characteristics:			
Gestational Age (wk)	38.5 (38.0; 39.3)	37.0 (34.3;38.0)	< 0.001
BMI (kg/m^2)	29.2 (27.2; 30.8)	29.8 (26.8; 33.0)	0.15
Age (y)	30.4 ± 5.7	29.7 ± 5.2	0.61
Blood Pressure (mm Hg):			
Systolic	119.9 ± 11.5	155.0 ± 14.9	< 0.001
Diastolic	69.0 ± 7.2	97.4 ± 6.3	< 0.001
Fetal characteristics:			
Birth weight (kg)	3.4 (3.0; 3.7)	2.6 (1.9; 3.1)	< 0.001
SGA (n)	0 (0%)	6 (13.6%)	< 0.01

BMI – Body Mass Index; **SGA** – Small for Gestational Age

Table 2 – PAI-1, tPA and D-dimer data in maternal and fetal blood, in normal and preeclamptic cases

	Maternal		P	Fetal		P
	Normal (n = 42)	PEc (n = 44)		Normal (n = 40)	PEc (n = 44)	
D-dimer (ng/ml)	538.2 (391.2; 822.8)	488.5 (313.0;1091.3)	0.99	200.4 (101.6; 492.5)	190.7 (104.1; 588.3)	0.96
PAI-1 (ng/ml)	119.1 (86.8; 177.4)	173.8 (119.8; 211.3)	0.003	99.7 (52.2; 175.7)	58.3 (36.9; 123.1)	0.10
tPA (ng/ml)	9.7 (7.3; 13.3)	20.9 (14.0; 27.0)	<0.001	3.4 (2.2; 5.3)	4.8 (3.1; 10.1)	0.02
PAI-1/tPA	11.9 (8.4; 14.5)	7.6 (5.7; 10.4)	<0.001	23.0 (11.3; 68.1)	9.6 (4.5; 35.2)	0.006
Platelets (x10^9/l)	178.0 (142.0; 203.5)	197.5 (141.0; 238.5)	0.25	271.0 (210.3; 299.8)	218.0 (200.5; 271.0)	0.056

PEc, Preeclamptic

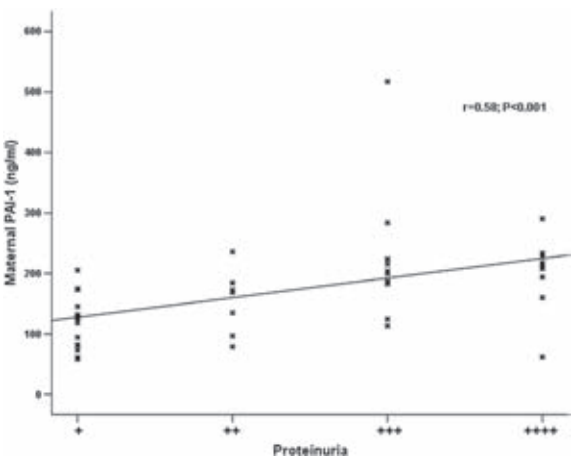

Figure 1 – Proteinuria *vs* maternal PAI-1.

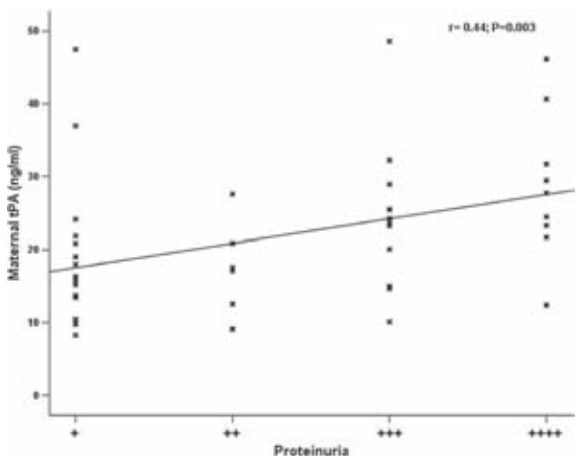

Figure 2 – Proteinuria *vs* maternal tPA.

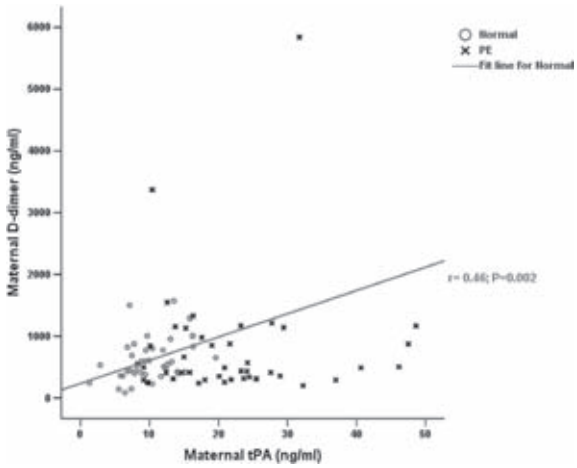

Figure 3 – Maternal tPA *vs* maternal D-dimer.

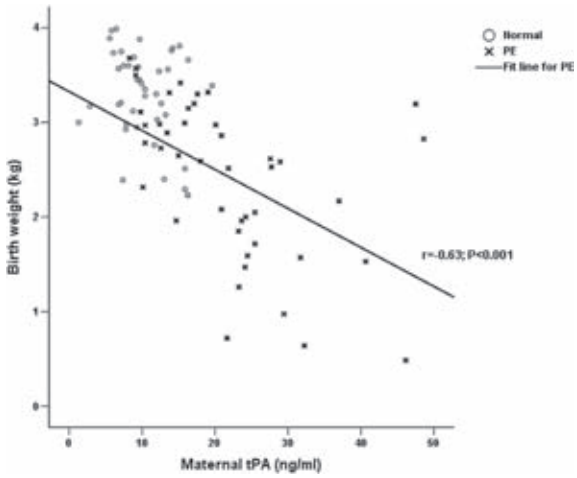

Figure 4 – Maternal tPA *vs* birth weight.

DISCUSSION AND CONCLUSION

Our data suggest that the significant rise of tPA and PAI-1 in PEc women, when compared with normal pregnant women, may reflect endothelial dysfunction. Similar findings for tPA were observed in PEc newborns, suggesting also some degree of endothelial dysfunction. Actually, a significant positive correlation was observed between mother's and newborn's tPA values, suggesting that the endothelial dysfunction occurring in mothers is linked to the same disturbance in their newborns.

Moreover, tPA and PAI-1 may provide markers of the severity of PE, considering their significant positive correlations with proteinuria.

In summary, tPA and PAI-1 levels are higher in PEc women, suggesting endothelial dysfunction, and correlate with the severity of PE. Furthermore, these PEc hemostatic changes seem to have impact in fetal circulation. We suggest that tPA may be a good marker of fibrinolytic impairment and of endothelial dysfunction, particularly in the maternal circulation, and that the impact of raised tPA levels in the neonates from PEc mothers deserves further studies.

ACKNOWLEDGEMENTS

The authors are grateful to the nursery group of Obstetrics Service of Hospital S. João, in particular nurse Célia Ribeiro for blood sample collection.

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ALTERED ERYTHROCYTE MEMBRANE BAND 3 PROFILE IN CHRONIC RENAL FAILURE PATIENTS UNDER HAEMODIALYSIS

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ABSTRACT

Our aim was to study changes in RBC membrane band 3 profile, as a cumulative marker of RBC changes, in chronic renal failure (CRF) patients under haemodialysis and recombinant human erythropoietin (rhEPO) therapy and its linkage with resistance to this therapy.

We studied 63 CRF patients, 32 responders and 31 non-responders to rhEPO therapy, and 26 healthy individuals matched for age and gender. We evaluated the band 3 profile and membrane-bound haemoglobin (MBH). Total serum bilirrubin, glutathione peroxidase (GPx) and superoxide dismutase activities, RBC count, haematocrit, haemoglobin concentration, haematimetric indices and reticulocyte were also evaluated. CRF patients presented anaemia, slightly regenerative, as showed by the decreased RBC count, Hb and haematocrit, alongside with an increased reticulocyte count, RPI and RDW values. CRF patients showed a statistically significant decrease in high molecular weight aggregates and proteolytic fragments (Pfrag), and a rise in Band 3 monomer. A rise in GPx and a trend to lower values of MBH were also found in CRF patients. A positive correlation was found between Pfrag and, Hb and haematocrit. When comparing the haematological data between the two groups of CRF patients,

we found that non-responders patients were more anaemic, and presented a statistically significant decrease in Pfrag, and a trend for a rise in MBH, suggesting a higher RBC damage.

Our data suggest that band 3 profile seems to be a good marker of erythrocyte changes in CRF patients. These changes seem to be associated with a younger RBC population, but also with a rise in RBC damage, which is enhanced in non-responders CRF patients. Band 3 profile could be used as a marker of RBC changes in these patients and in the understanding of the mechanism of resistance to rhEPO therapy.

Key-Words: Chronic renal failure, Band 3, rhEPO, Erythropoietin.

INTRODUCTION

Band 3 protein is the major integral protein of the red blood cell (RBC) membrane. It is known as the senescent neoantigen, as modifications in band 3 protein, by proteolytic cleavage, clustering or exposure of unusual epitopes, trigger the binding of specific anti-band 3 autoantibodies and complement activation, marking RBC for death. An abnormal band 3 profile [an increase in high molecular weight aggregates (HMWAg) and a decrease in band 3 monomer and proteolytic fragments (Pfrag)], has been associated with RBC damage/aging in inflammatory conditions associated with oxidative stress, namely in cardiovascular disease, pregnancy and acute physical exercise[1-3].

Chronic renal failure (CRF) has also been associated with both inflammation and oxidative stress. A deficient renal erythropoietin secretion underlies the development of an anaemia, which is usually corrected by therapy with recombinant human erythropoietin (rhEPO). However, about 25% of the patients do not respond to this therapy[4].

Our aim was to study the erythrocyte membrane band 3 profile, as a cumulative marker of RBC changes, in CRF patients under haemodialysis and rhEPO therapy.

MATERIALS AND METHODS

SUBJECTS AND SAMPLES

We studied 89 individuals including 63 CRF patients, 32 responders and 31 non-responders to rhEPO therapy, and 26 healthy controls. The rhEPO maintenance dose for responder's patients was 8.03 ± 5.97 U/Kg/week/Hb and for non-responders was 56.70 ± 22.40 U/Kg/week/Hb. The two groups of patients were matched for age, gender, weight, body mass index, mean time under haemodialysis, urea reduction ratio, urea Ktv and parathyroid hormone serum levels. No laboratory indicators of iron deficiency and/or vitamin B12 and folate deficiencies were found in CRF patients

Peripheral blood samples were collected into EDTA containing tubes.

The causes of renal failure in patient's population were as follows: diabetic nephropathy (n=19), chronic glomerulonephritis (n=10), polycystic kidney disease (n=3), hypertensive nephrosclerosis (n=3), obstructive nephropathy (n=3), pyelonephritis associated with neurogenic bladder (n=1), nephrolithiasis (n=1), chronic interstitial nephritis (n=1), Alport syndrome (n=1), renal vascular disease due to polyarteritis (n=1) and chronic renal failure of uncertain aetiology (n=17).

Patients with autoimmune disease, malignancy, haematological disorders, and acute or chronic infection were excluded. All patients gave their informed consent to participate in this study. Classification of CRF patients in responders or non-responders, was performed in accordance with the European Best Practice Guidelines (5), that defines resistance to rhEPO as a failure to achieve target haemoglobin levels with doses of epoetin more than 300 IU/Kg/week or 1,5 mg/Kg/week of darbopoietin-alfa.

Age and gender-matched individuals, with normal haematological and biochemical values, without any history of renal or inflammatory disease, were used as controls.

In all individuals (patients and controls), we evaluated RBC count, haematocrit, haemoglobin concentration (Hb), haematimetric indices, red cell distribution width (RDW) (by using a blood cell counter); reticulocyte count (brilliant cresyl blue staining), reticulocyte production index (RPI); membrane bound haemoglobin (MBH) (by spectrophotometry)[1], total serum bilirubin levels, glutathione peroxidase (GPx) (RANSEL, Randox, UK) and superoxide dismutase (SOD) activities (RANSOD, Randox, UK); band 3 profile [% of band 3 monomer, high molecular weight aggregates (HMWAg) and proteolytic fragments (Pfrag)].

Band 3 profile

RBC membranes were treated with an equal volume of a solubilisation buffer containing 0.125M Tris HCl pH 6.8, 4% sodium dodecil sulfate (SDS), 20% glycerol, and 10% 2-mercaptoethanol, heat-denatured and submitted to polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous Laemmli system. Membrane proteins were electrophoretically transferred from SDS gels to a nitrocellulose sheet. Additional reactive sites on the nitrocellulose were blocked by incubation in 5% low fat dry milk and 0.1% Triton-X 100 in PBS (phosphate buffered saline) pH 7.0, for overnight at 4ºC and under gentle rotation. Band 3 immunoblot was performed; monoclonal antibodies anti-human band 3, produced in mouse, recognising an epitope located in the cytoplasmic pole of the band 3 molecule (Sigma), were added (dilution 1:3000) and incubated for 4 h; the washing of the nitrocellulose was followed by the addition and incubation with antimouse Ig peroxidase linked (Sigma) for 1 h (dilution 1 : 4000). The incubations were carried out at room temperature; the dilutions of the antibodies were prepared with PBS pH 7.0 containing 0.1% Triton-X 100 and 0.5% low fat dry milk (9,10). In the washes, the same buffer without low fat dry milk was used. Hydrogen peroxide and α-cloronaphtol were used to develop the immunoblot. The band 3 immunoblots were scanned (DarkroomCN UV/wl, BiocaptMW version 99, Vilbert Lourmat) and quantified by densitometry (Bio 1D++version 99, Vilbert Lourmat).

Data analysis

Statistical analyses were carried out using the *SPSS* package. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey´s honestly significant difference (HSD) *post hoc* test. For data not normally distributed, differences between the three groups were evaluated by the Kruskal-Wallis test; for single comparisons (two groups), the Mann-Whitney *U* test was used. Significance was accepted at p less than 0.05.

RESULTS

CRF patients presented anaemia, slightly regenerative, as showed by the decreased RBC count, Hb and haematocrit, alongside with an increased reticulocyte count, RPI and RDW values. A rise in GPx and a trend to lower values of MBH were also found in CRF patients (Table I). CRF patients showed a statistically significant decrease in HMWAg and Pfrag, and a rise in Band 3 monomer (Table I; Fig. 1). A positive correlation was found between Pfrag and Hb and haematocrit (Fig. 2). When comparing the haematological data between the two groups of CRF patients, we found that non-responders patients were more anaemic, and presented a statistically significant decrease in Pfrag, and a trend for a rise in MBH, suggesting a higher RBC damage.

Table I – Haematological and biochemical data for controls and CRF patients – responders and non-responders to rhEPO therapy.

	Controls (n=26)	All patients (n=63)	Responders (n=32)	Non-responders (n=31)
Hb (g/dL)	13.90 (13.2-15.00)	10.90 (10.30-12.30) b)	11.70 (10.83-12.68) b)	10.4 (9.00-11.30) b) c)
Haematocrit (%)	43.10 (40.10-46.70)	34.20 (30.60-37.10) b)	35.15 (32.25-38.35) b)	31.10 (27.70-35.20) b) c)
RBC ($x10^{12}$/L)	4.72 ± 0.59	3.68 ± 0.54 b)	3.76 ± 0.42 b)	3.58 ± 0.64 b)
MCV (fL)	92.00 (90.00-94.00)	93.80 (90.00-98.20) a)	95.80 (92.48-98.08) a)	92.30 (85.40-100.30)
MCH (pg)	29.83 ± 1.39	30.15 ± 3.04	31.29 ± 1.53 b)	28.97 ± 3.73 c)
MCHC (g/dL)	32.48 ± 0.58	32.03 ± 2.37	33.16 ± 1.77	30.85 ± 2.35 a)c)
RDW (%)	12.79 ± 0.52	15.92 ± 2.56 b)	14.56 ± 1.23 b)	17.32 ± 2.83 b)c)
RBC production / damage / removal				
Reticulocytes ($x10^9$ /L)	33.57 ± 22.78	61.03 ± 31.36 b)	55.12 ± 30.98 a)	67.14 ± 31.06 b)
RPI	0.42 (0.19-0.66)	0.98 (0.58-1.40) b)	1.08 (0.72-1.51) b)	0.92 (0.52-1.24) a)
Total Bilirubin (mg/dL)	0.62 ± 0.25	0.61 ± 0.24	0.61 ± 0.23	0.62 ± 0.24
MBH ($x10^{-4}$ %)	53.00 (37.75-89.75)	50.00 (28.00-82.00)	45.50 (25.25-80.75)	58.50 (30.50-100.75)
SOD (IU/g Hb)	1039.8 (737.4-1331.6)	898.6 (679.4-1454.2)	858.97 (662.4-1256.5)	1074.76 (581.6-2638.7)
GPx (IU/g Hb)	35.62 ± 8.83	45.82 ± 13.69 a)	48.73±13.46 a)	43.11±13.87
Band 3 profile				
HMWAg (%)		15.23 (13.38-19.40) a)	14.86 (11.30-20.19) a)	15.92 (14.28-18.68) a)
Band 3 monomer (%)		61.84 (56.87-64.41) b)	61.26 (56.08-65.06) a)	62.17 (58.01-64.29) b)
Pfrag (%)		22.70 ± 6.01 a)	24.01 ± 6.03	21.34 ± 5.78 a) c)

a) $p<0.05$, *vs* controls; b) $p<0.001$, *vs* controls; c) $p<0.05$ *vs* responders.

DISCUSSION

Our data suggest that band 3 profile seems to be a good marker of erythrocyte changes in CRF patients. These changes seem to be associated with a younger RBC population, but also with a rise in RBC damage, which is enhanced in non--responders CRF patients. Band 3 profile could be used as a marker of RBC changes in these patients and in the understanding of the mechanism of resistance to rhEPO therapy.

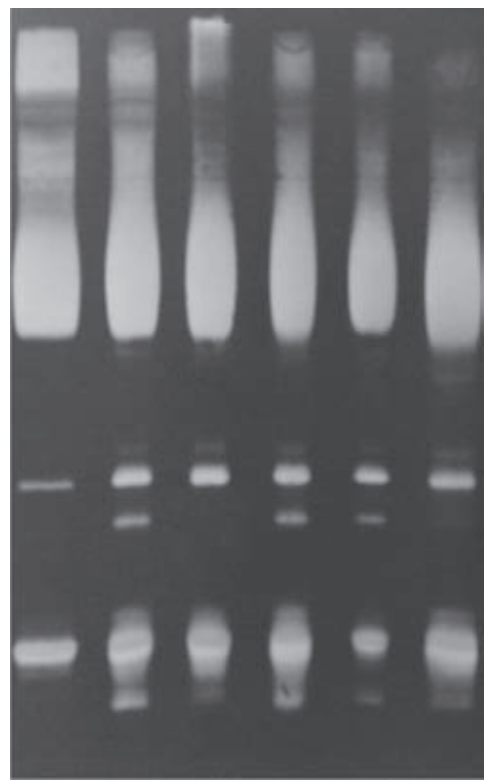

Fig. 1 – Illustration of Band 3 profiles. C1, C2 – Controls; P1 and P2 – responders CRF patients; P3, P4 – non-responders CRF patients.

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We are very grateful to FMC, Dinefro – Diálises e Nefrologia, SA and Uninefro – Sociedade Prestadora de Cuidados Médicos e de Diálise, SA, and to their nurses for the technical support. This study was supported by a PhD grant (SFRH/BD/27688/2006) attributed to E. Costa by FCT and FSE.

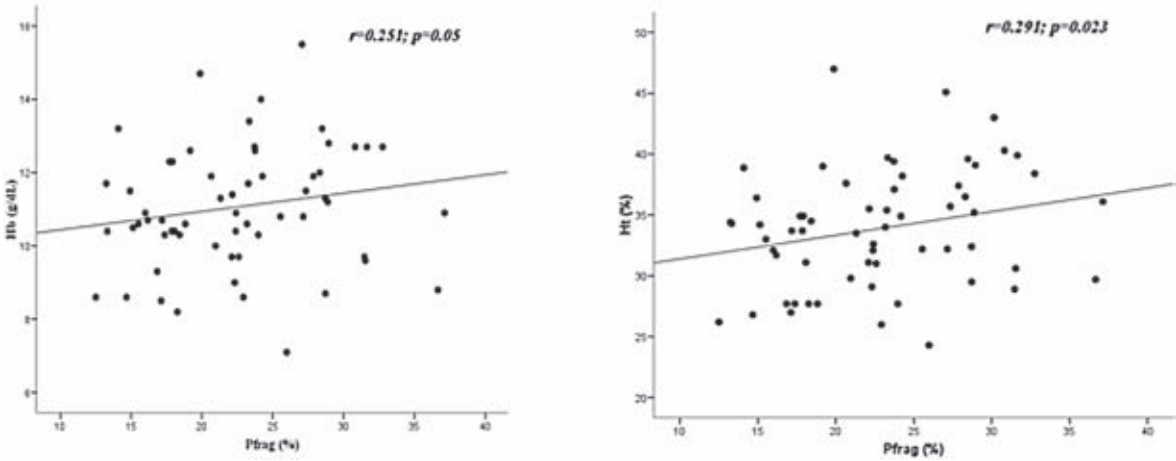

Fig. 2 – Correlation of Pfrag with Hb and haematocrit (Ht) in CRF patients.

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FIBRINOLYTIC ACTIVITY AND VASCULAR ACCESS IN CHRONIC RENAL FAILURE PATIENTS UNDER HAEMODIALYSIS

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ABSTRACT

Our aim was to study the relationship between fibrinolytic activity and the type of vascular access in haemodialysis patients. We measured the circulating antigen levels of plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (tPA) and D-dimers. This study was performed in 50 CRF patients under regular haemodialysis, 11 with central venous dialysis catheter and 39 with AV-fistula, and in 25 healthy controls.

Compared with controls, CRF patients presented significantly lower levels of tPA and with higher levels of D-dimers. In CRF patients, the levels of D-dimers correlated positively and significantly ($r=0.359$, $p=0.01$) with rhEPO doses (rhEPO/Kg/week) and negatively with haemoglobin levels ($r=-0.335$, $p=0.017$). When comparing the two groups of CRF patients, we found that those with central venous catheter vascular access presented a statistical significant rise in D-dimer and tPA plasma levels. No difference was found between the two groups of patients concerning the plasma levels PAI-1.

Our results showed an altered haemostasis in CRF patients, as suggested by the rise in D-dimer, an index of fibrin turnover and intravascular thrombogenesis. The increased levels of D-dimer and tPA in CRF patients, particularly in those using central venous dialysis catheters, led us to propose a relationship between the type of vascular access chosen for the haemodialysis procedure, and the risk of thrombogenesis. It seems reasonable to assume that these patients present an increased risk for cardiovascular disease events.

Key-Words: fibrinolytic activity, Chronic Renal Failure, Vascular access, D-dimers.

INTRODUCTION

Cardiovascular disease events are the main cause of death in chronic renal failure (CRF) patients. Disturbances in coagulation and fibrinolysis have been reported in patients with chronic uremia, and are known to contribute to the pathogenesis of cardiovascular diseases. However, studies about coagulation and fibrinolysis in CRF patients under regular hemodialysis have yielded conflicting results. Some studies reported a suppressed fibrinolysis and others an increased fibrinolysis[1-3]. These controversial results may be related to the type of vascular access (central venous dialysis catheter or AV-fistula) chosen for the haemodialysis procedure, and may associate a different risk of thrombosis[3-5].

Our aim was to study the relationship between fibrinolytic activity and the type of vascular access in haemodialysis patients.

MATERIALS AND METHODS

Subjects

We studied 50 CRF patients under regular haemodialysis (32 males, 18 females; mean age 64.5 ± 15.4), 11 with Central Venous Dialysis Catheter and 39 with AV-Fistula.

CRF patients were under therapeutic haemodialysis three times per week, for 3 to 5 h, for a median period of time of 36 months. All patients used the high-flux polysulfone FX-class dialysers of Fresenius, 25 with FX60, 23 with FX80 and 2 with FX100 dialyser type.

The causes of renal failure in patient's population were as follows: diabetic nephropathy (n=16), chronic glomerulonephritis (n=6), polycystic kidney disease (n=5), hypertensive nephrosclerosis (n=3), obstructive nephropathy (n=3), pyelonephritis associated with neurogenic bladder (n=1), nephrolithiasis (n=1), chronic interstitial nephritis (n=1), Alport syndrome (n=1), renal vascular disease due to polyarteritis (n=1) and chronic renal failure of uncertain aetiology (n=12). Patients with autoimmune disease, malignancy, haematological disorders, and acute or chronic infection were excluded. All patients gave their informed consent to participate in this study.

The control group included 25 healthy volunteers presenting normal haematological and biochemical values, with no history of renal or inflammatory diseases, and, as far as possible, age and gender matched with CRF patients.

Assays

Plasma levels of plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (tPA) and D-dimers were evaluated by enzyme immunoassays (TintElize PAI-1, TintElize tPA and TintElize D-dimer, Biopool-Trinity Biotech Company, respectively).

Data analysis

For statistical analysis, we used the Statistical Package for Social Sciences, version 14.0. Kolmogorov Smirnov statistics were used to evaluate sample normality distribution. Comparisons between groups were performed using Kruskal-Wallis test and Mann-Whitney U (data with a non-Gaussian distribution) or Student's t-test (data with a Gaussian distribution). Spearman´s rank correlation coefficient was used to evaluate relationships between sets of data. Significance was accepted at p less than 0.05.

RESULTS

Compared with controls, CRF patients presented significantly lower levels of PAI-1 and with higher levels of D-dimers (Fig. 1). In CRF patients, the levels of D-dimers correlated positively and significantly (r=0.359, p=0.01) with rhEPO doses (rhEPO/Kg/week) and negatively with haemoglobin levels (r=-0.335, p=0.017) (Fig.2). When com-

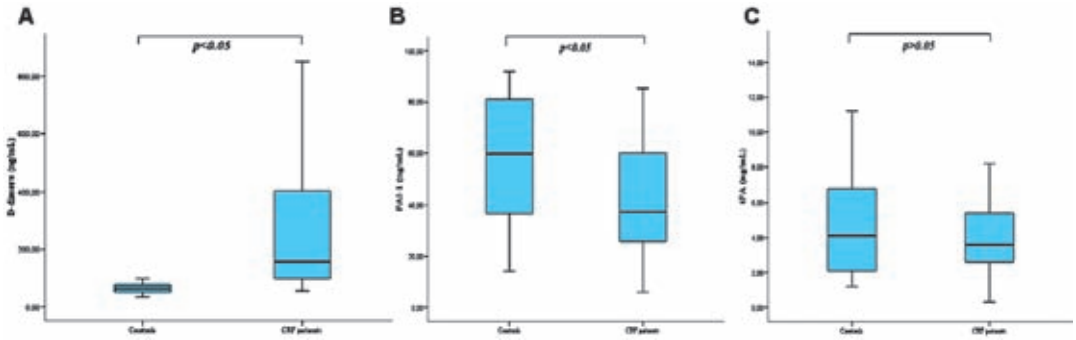

Fig. 1 – Compared with controls, CRF patients presented significantly higher levels of D-dimers (A) and lower levels of PAI-1 (B). No difference between these two groups was found for tPA levels (C).

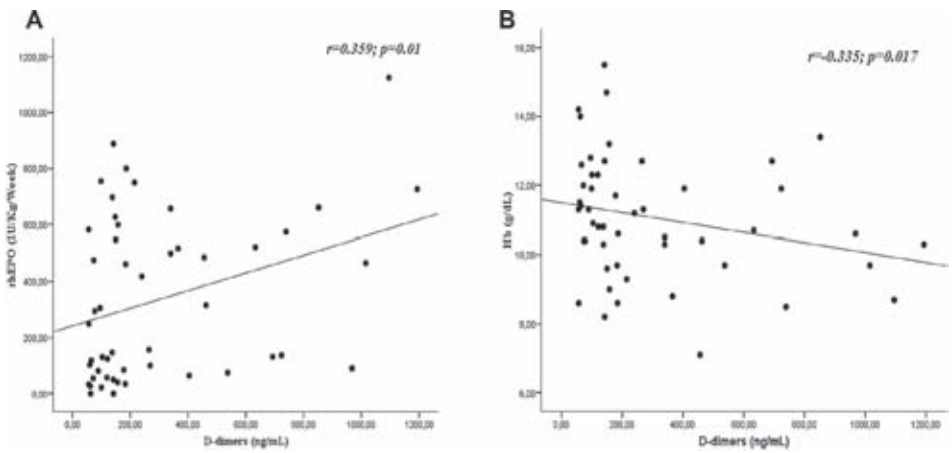

Fig. 2 – Correlation between D-dimers levels and rhEPO doses (A) and hemoglobin levels (B) in CRF patients.

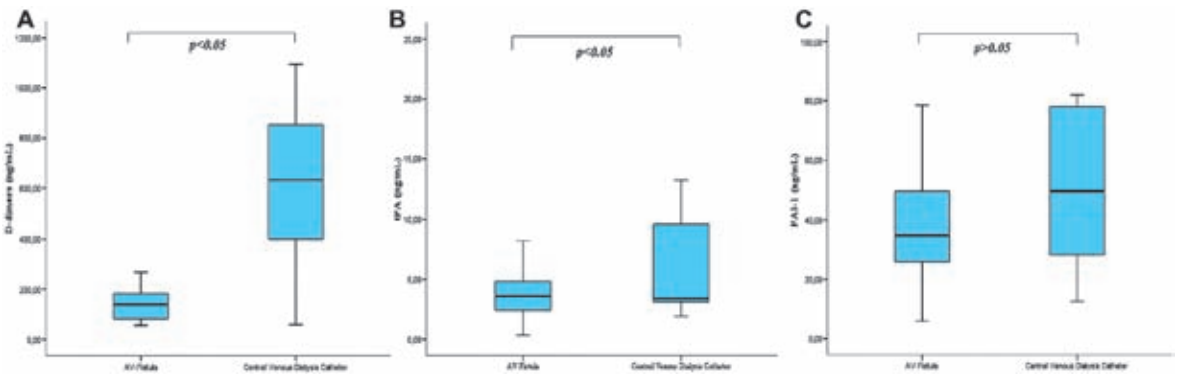

Fig. 3 – CRF patients with Central Venous Catheter as vascular access presented a statistical significant rise in D-dimers (A) and tPA (B) plasma levels, when compared with those using AV-Fistula as vascular access. No difference was found between the two groups of patients concerning the plasma levels of PAI-1 (C).

paring the two groups of CRF patients, we found that those with central venous catheter vascular access presented a statistical significant rise in D-dimer and tPA plasma levels. No difference was found between the two groups of patients concerning the plasma levels PAI-1 (Fig.3).

DISCUSSION

Our results showed an altered haemostasis in CRF patients, as suggested by the rise in D-dimer, an index of fibrin turnover and intravascular thrombogenesis. The increased levels of D-dimer and tPA in CRF patients, particularly in those using central venous dialysis catheters, led us to propose a relationship between the type of vascular access chosen for the haemodialysis procedure, and the risk of thrombogenesis. It seems reasonable to assume that these patients may present an increased risk for cardiovascular disease events.

ACKNOWLEDGEMENTS

We are very grateful to FMC, Dinefro – Diálises e Nefrologia, SA and Uninefro – Sociedade Prestadora de Cuidados Médicos e de Diálise, SA, and to their nurses for the technical support. This study was supported by a PhD grant (SFRH/BD/27688/2006) attributed to E. Costa by FCT and FSE.

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EFFECTS OF ACETYLCHOLINE ON NO TRANSLOCATION IN ABNORMAL AND MANIPULATED RED BLOOD CELLS

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ABSTRACT

Nitric oxide (NO) is known to be an important vasodilator messenger interfering in a number of physiological and pathophysiological processes. We have verified that in some diseases red blood cells have hemorheological dysfunctions, such as, changes on erythrocyte deformability and erythrocyte aggregation. We also demonstrated that the presence of acetylcholine (ACh, natural substrate of acetylcholinesterase enzyme) induce changes on the healthy erythrocytes vasomodulation role. The aim of this study was to access the ex vivo response of NO translocation in abnormal red blood cells after ACh 10 μM stimulation and to know if this action could be related to intraglobular nitrosylated molecules and phosphorylated/dephosphorylated protein band 3 by, tyrosine kinase and phosphatase proteins, respectively. For this propose we determined NO concentration, by an amperometric method on in vitro erythrocytes suspensions from patients with different types of diseases (drepanocytosis (n=9), renal transplantation (n=36), chronic venous peripheral disease (n=22), arterial hypertension (n=20) hypercholesterolemia (n=102) coronary ischemia (n=34) and delirium associated with stroke (n=115) comparing with NO levels achieved on erythrocytes of healthy persons (n=27). We stimulate erythrocytes of healthy persons with tyrosine kinase and tyrosine phosphatase proteins inhibitors ($p72^{syk}$ inhibitor 10 μM and calpeptin 10 μM, respectively) for 30 minutes at 37 ºC and also measured NO levels. NO concentration values obtained were: 2,0 ± 0,8 nM (control), 4,5 ± 1,4 nM (drepanocytosis, P<0,001), 2,2 ± 0,9 nM (renal transplantation), 2,3 ± 0,6 nM (chronic venous peripheral disease), 2,8 ± 1,1 nM (hypertension, P=0,005), 2,3 ± 0,7 nM (hypercholesterolemia), 2,8 ± 0,9 nM

(coronary ischemia, P=0,001) and 2,6 ± 0,8 nM (delirium associated with stroke, P=0,001). We observed the most significant change on NO translocation with drepanocytosis erythrocytes samples which could be a positive factor for the compromised tissue oxygenation in this kind of anaemia. On all different pathologies studied there were a tendency to increase NO erythrocyte translocation. On the other and, manipulated red blood cells shows that in the presence of ACh, forming an acetylcholinesterase active enzyme complex, increase NO levels. The addition of $p72^{syk}$ inhibitor, protein band 3 at partially phosphorylated state, reveal a decrease of NO concentrations with ACh. In presence of calpeptin and ACh, band 3 being totally phosphorylated, we observed an increase of NO levels.

In conclusion, human erythrocytes of different diseases have diverse physiological responses to ACh stimulation that leads to changes on NO mobilization mechanisms. When we manipulated the phosphorylated/dephosphorylated states of band 3 protein, the results reveal that there are changes on erythrocytes NO translocation in the presence of acetylcholinesterase substrate. The results demonstrated the key-role of acetylcholinesterase effector-associated band 3 phosphorylation/dephosphorylation pathway in the mobilization of the erythrocyte NO stores, which might facilitate to understand some intracellular erythrocyte-dependent events underlying hypoxic conditions on microcirculation disease. This suggests a future target for vasodilator therapeutic action on a microcirculatory network, by controlling the tissue oxygenation, that could be damaged by different sorts of stimulus.

INTRODUCTION

Several endothelium-derived substances have been isolated causing smooth muscle relaxation. Nitric oxide is one of them and its biosynthesis pathway is well characterized[1,2]. Nitric oxide is a relatively stable gas, with ability to diffuse through the cell membrane[2]. Reaction of vascular-derived NO with hemoglobin is believed to be the most important pathway for limiting NO bioactivity. The reaction of the iron-containing heme groups of oxy- and deoxyhemoglobin with NO produces, respectively, methemoglobin and nitrate ions and iron-nitrosyl-hemoglobin[3]. The process of NO diffusion through the cell membrane followed by diffusion through plasma and the chemical reaction with erythrocyte internal hemoglobin indicates that changes in membrane permeability and in other hemorheological parameters could occur[4-6].

Erythrocytes may release NO bound to hemoglobin in the microcirculation under low oxygen tension[7]. In 1980, Furchgott and Zawadzki reported that acetylcholine (ACh) causes relaxation of isolated blood vessels only in presence of an intact endothelial cell layer[8]. Acetylcholine infusions to forearm blood flow are used to evaluate the endothelial cells layers function[9]. This vasomotor response is NO dependent and the loss of its biological activity and biosynthesis is one of the mechanisms responsible of endothelial dysfunction. Depressed endothelium dependent vascular relaxation is present in patients with essential hypertension[10,11]. Atherosclerosis, hypertension, diabetes, reperfusion injury and vasculopathy as well as angioplasty, bypass surgery, and transplantation are examples of diseases where NO insufficiency induces endothelium dysfunction[12].

We have recently disclosed an increased NO metabolism in acetylcholine-treated erythrocytes[13]. Acetylcholine (ACh) is the natural substrate of the erythrocyte membrane-bound acetylcholinesterase, which function still remains scarcely stated albeit the well-known existence of more active (acetylcholine-AChE) versus less active

(velnacrine-AChE) enzymatic complexes[14]. Velnacrine maleate (VM) has been described in red cells as an acetylcholinesterase inhibitor. VM ability to strengthen cytoskeletal protein-protein interactions in erythrocyte membranes illustrates its relevance as a strong therapeutic weapon against Alzheimer's disease[15]. We hypothesize the involvement of AChE effectors enzymatic complexes in a band 3-dependent signal transduction pathway related to changes in the intracellular NO metabolism and the production of its chief metabolites, nitrites and nitrates[13,16].

This study was designed to assess the ex vivo response of NO translocation in abnormal red blood cells after ACh 10 μM stimulation and to know if this action could be related to intraglobular nitrosylated molecules and phosphorylated/dephosphorylated protein band 3 by, tyrosine kinase and phosphatase proteins, respectively.

EXPERIMENTAL DESIGN

Venous blood samples were collected from healthy humans (n=27) and patients with sickle cell disease (n=9), renal transplantation (n=36), chronic venous peripheral disease (n=22), arterial hypertension (n=20), hypercholesterolemia (n=102), coronary ischemia (n=34) and delirium associated with stroke (n=115). Erythrocyte suspensions from healthy humans were performed with addition of sodium chloride 0,9%, pH 7,4, to reconstitute the initial hematocrit (Ht, 45 %). Aliquots were incubated 30 minutes at 37ºC, with AChE effectors (either with ACh 10 μM or VM (acetylcholinesterase inhibitor, 10 μM)), in the presence and absence of $p72^{syk}$ inhibitor 10 μM and PTP inhibitor (calpeptin 10 μM). For amperometric NO quantification we used an amiNO-700 sensor (Innovative Instruments Inc.

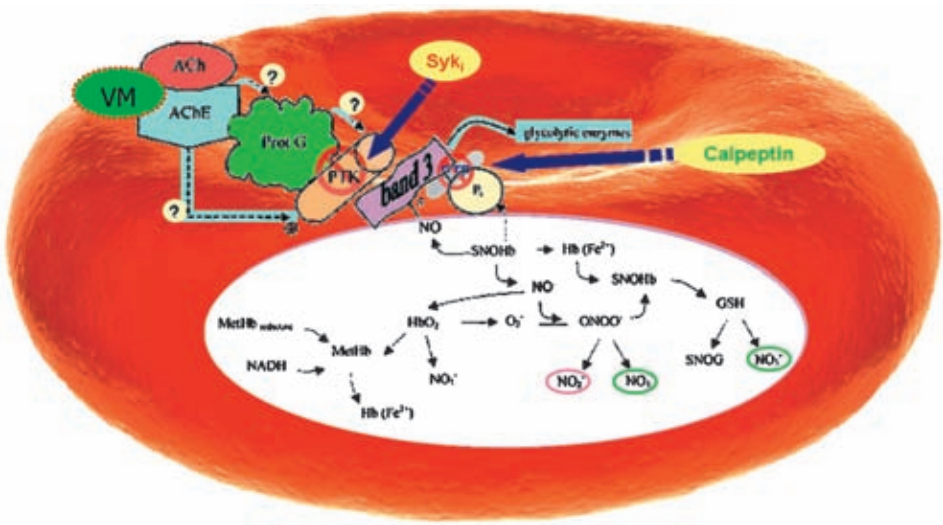

Fig. 1 – A hypothesis for acetylcholinesterase role on signal transduction mechanism in response to action of ACh or VM on NO production (and its metabolites) in human erythrocytes suspensions. Transnitrosilation process between phosphorylated / dephosphorylated band 3 and SNOHb could be associated to an unidentified mechanism mediated by formation of AChE-ACh (more active) or AChE-VM (less active) complexes, when PTK or PTP enzymatic activity is inhibited. This process could also be dependent of a G protein[13].

Abbreviations: ACh (acetylcholine); **VM** (velnacrine maleate); **AChE** (acetylcholinesterase); **Prot G** (G protein); **PTK** (protein tyrosine kinase); **PTP** (protein tyrosine phosphatase); **Pi** (phosphate); **NADH** (nicotinamide adenine dinucleotide, reduced); **Hb** (hemoglobin); **SNOHb** (S-nitrosohemoglobin); **NO** (nitric oxide); $\mathbf{O_2^-}$ (anion peroxide); $\mathbf{ONOO^-}$ (peroxinitrite); $\mathbf{NO_3^-}$ (nitrate); $\mathbf{NO_2^-}$ (nitrite); **GSH** (glutatione reductase); **SNOG** (S-nitrosothiol); $\mathbf{HbO_2}$ (oxyhemoglobin); **MetHb** (methemoglobin); **Calpeptin** (PTP inhibitor) **Syki** (PTK inhibitor)

FL, USA)[17]. Erythrocyte suspensions (Ht= 0.05%) were incubated for 15 minutes (room temperature) and stimulated with ACh 10 μM for monitorized erythrocytic nitric oxide mobilization. All the subjects gave their informed consent to participate in this study. All patient samples were tested against the control. Statistical significance was considered for values of $P < 0.05$. The results are presented as means ± SD (standard deviation).

RESULTS AND CONCLUSIONS

From Fig. 2 we observe that, after acetylcholine stimulation, there is a significant increase of nitric oxide mobilization on erythrocytes from patients with sickle cell disease, arterial hypertension, coronary ischemia and delirium associated with stroke. Erythrocytes from patients with renal transplantation, chronic venous peripheral disease and hypercholesterolemia also show a tendency to increase nitric oxide mobilization. In patients with sickle cell disease nitric oxide seems to participate in the compensatory response to chronic vascular injury.

From these results we may conclude that vascular pathologies have different physiological responses to acetylcholine stimulation and show differences on nitric oxide mobilization mechanisms.

NO concentration values achieved in control samples were of 1.09 ± 0.30 nM. ACh-stimulated erythrocytes (ACh 10 μM) significantly increased the control value (2.05 ± 0.29 nM vs NO control value; $P < 0.001$) and the augment verified with VM 10 μM stimulation was not significant (1.27 ± 0.31 nM vs NO control value) (vd Fig. 3). From Fig. 3 we can see that both calpeptin (1.69 ± 0.31 nM, $P<0.01$) and $p72^{syk}$ inhibitor (1.50 ± 0.29 nM, $P<0.01$) significantly increase the NO levels obtained in the control samples. The presence of $p53/56^{lyn}$ inhibitor did not increase the NO concentration (1.14 ± 0.31 nM, ns).

Erythrocytes incubation with both $p72^{syk}$ and $p53/56^{lyn}$ inhibitors seems to decrease the NO levels, although the results were not statistically significant (0.81 ± 0.30 nM) (vd Fig. 3). When compared to the samples with calpeptin and $p72^{syk}$ inhibitor, ACh decreases in the NO concentration. The opposite was verified in erythrocytes incubation with $p53/56^{lyn}$ inhibitor. As to VM, the same

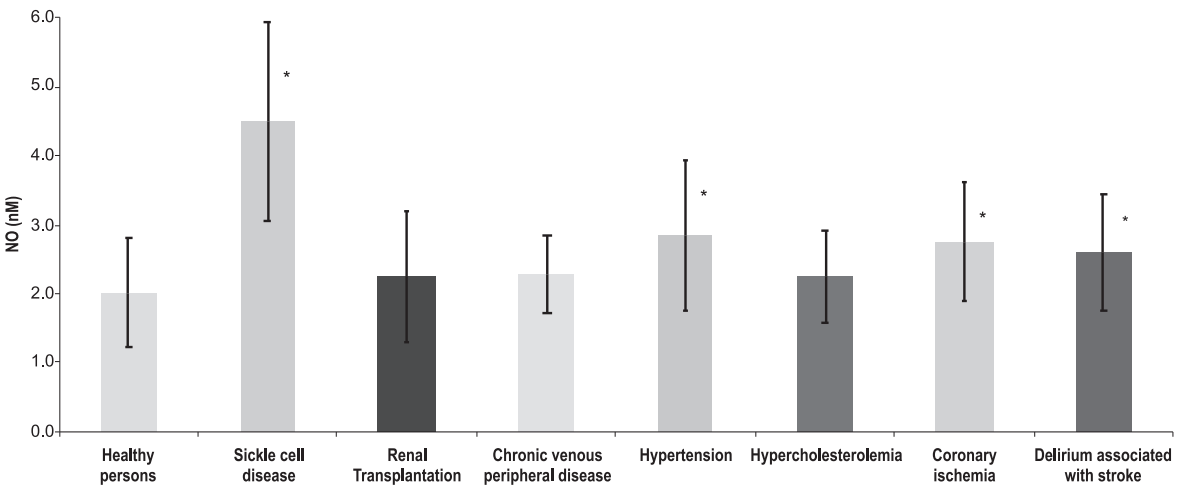

Fig. 2 – Changes on NO mobilization from erythrocytes suspensions incubated with ACh 10μM of control (healthy persons) and patients with sickle cell disease, renal transplantation, chronic venous peripheral disease, arterial hypertension, hypercholesterolemia, coronary ischemia and delirium associated with stroke. Values are in mean ± SD**.** * $P < 0.001$ for sickle cell disease, coronary ischemia and delirium; *$P = 0.005$ for arterial hypertension

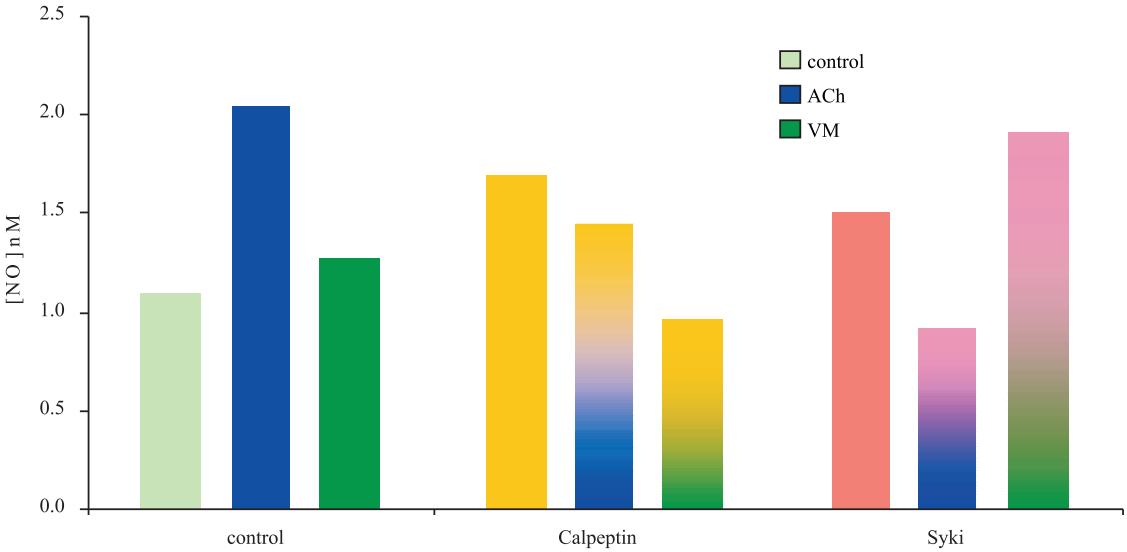

Fig. 3 – Changes of in vitro NO mobilization, after incubation of erythrocytes suspensions with ACh 10 µM, VM 10 µM, and in the absence or presence of Syk inhibitor (Syki) 10 µM, and calpeptin 10 µM.

Values are in mean, n = 4. Standard deviation values were not shown because is just about the same for every erythrocyte suspension. * P<0.01; ** P<0.001

decrease on NO levels was stated with calpeptin, although with $p72^{syk}$ and/or $p53/56^{lyn}$ inhibitors a statistically significant increase was documented (vd Fig.3).

In vitro manipulated erythrocytes (changes in band 3 protein phosphorylation and dephosphorylation) lead to different nitric oxide translocation when the cells were also in absence or in presence of acetylcholinesterase inhibitor/substrate. This fact could raise an hypothesis that acetylcholinesterase effectors associated with band 3 phosphorylation status could be a key-role in the mobilization of the erythrocytes nitric oxide stored.

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IDENTIFICATION OF THE LINKAGE BETWEEN G PROTEINS AND ERYTHROCYTE PROTEIN BAND 3

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ABSTRACT

Circulating acetylcholine (natural substrate of acetylcholinesterase) is able to modulate the microcirculatory blood flow by controlling nitric oxide (NO) intracellular mobilization, its metabolism (NO_x) and its release from erythrocytes. In reverse, velnacrine maleate plays a competitive role as an acetylcholinesterase (AChE) inhibitor decreasing NO-mediated erythrocyte responses.

In our previous studies we hypothesis that a possible response's mechanism lies on the NO translocation among nitrosylated molecules through a protein G linked to band 3 protein. Band 3 phosphorylated/dephosphorylated states are processed by major tyrosine-kinases (PTK) and phosphotyrosine-phosphatases (PTP).

So we intend to identify the G protein form that could be linked to the protein band 3 and to know each protein G sub-units (α,β,γ) are related to the activation or inhibition of acetylcholinesterase complex form and phosphorylation band 3 degree states. For this propose we made Western blotting analysis using primary antibodies to different protein G sub-units such as anti-protein G_β, anti-protein $G\alpha_{i1/2}$, anti-protein $G\alpha_{i3}$, anti-protein $G\alpha_{i3}/G\alpha_0$, anti protein $G\alpha_S$ and anti-protein $G\alpha_{q/11}$. We could identify on erythrocytes membrane soluble extracts possible linkage between protein $G\alpha_{i1/2}$ and/or protein G_β with protein band 3. The results were then confirmed by immunoprecipitation of this two protein G sub-units with following analysis by Western blot using antibodies against protein band 3 (C-terminal) and band 3 (N-terminal).

From all the blood samples aliquots studied we could concluded that G protein sub-units $G\alpha_{i1/2}$ and G_β could be linked with band 3 C-terminal site and only $G\alpha_{i1/2}$ are bonded with band 3 N-terminal. The connection between sub-unit G_β and band 3 at C-terminal was not seemed. Moreover when erythrocyte acetylcholinesterase was stimulated with acetylcholine and when is present with PTK inhibitors there was an increase of the expression of the linkage between $G\alpha_{i1/2}$ – Band 3 (C- and N-

terminal) and G_{β} - Band 3 (C –terminal). These two conformational states of G protein sub-units seem to be related with the phosphorylation band 3 protein states.

Heterotrimeric G proteins mediate signal transduction pathways; moreover it is known that stimulation of G_i results in inhibition of adenylyl cyclase and ATP release from these cells. So human erythrocyte $NO_{(x)}$ mobilization levels may occur under the influence of AChE effectors by mechanisms related to the degree of band 3-phosphorylation and activation of adenylyl cyclase. These events underlying NO translocation/mobilization changes may occur on microcirculation disease, a target upon which novel coadjuvant drugs may become accessible.

INTRODUCTION

We recently hypothesize the involvement of AChE effectors enzymatic complexes in a band 3-dependent signal transduction pathway related to changes in the intracellular NO metabolism and the production of its chief metabolites, nitrites and nitrates[1]. Our hypothesis is based on the acetylcholinesterase role on signal transduction mechanism in response to action of ACh or VM on NO production (and its metabolites) in human erythrocytes suspensions that could be

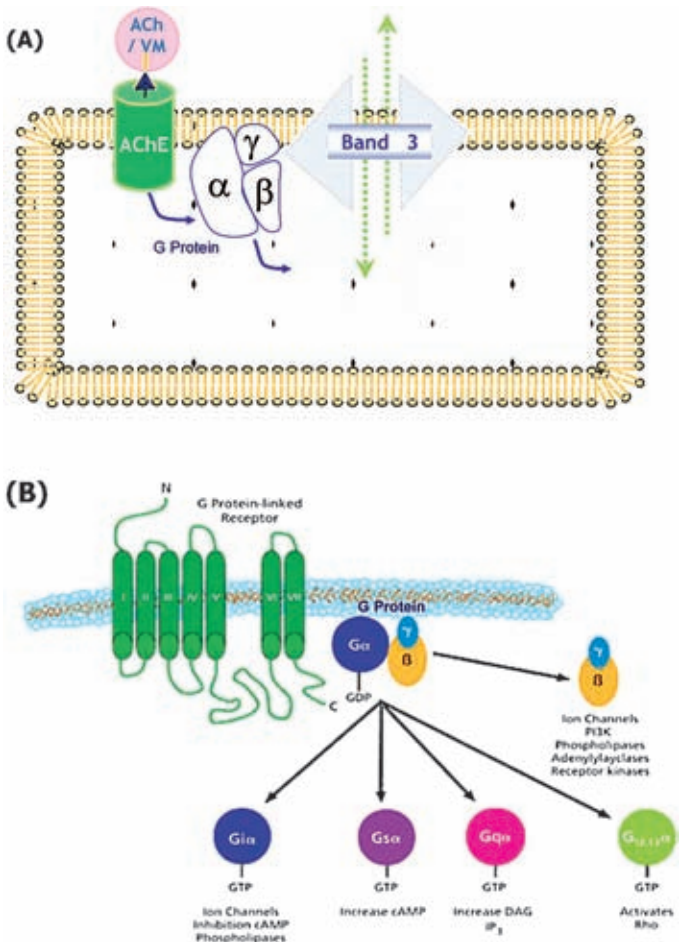
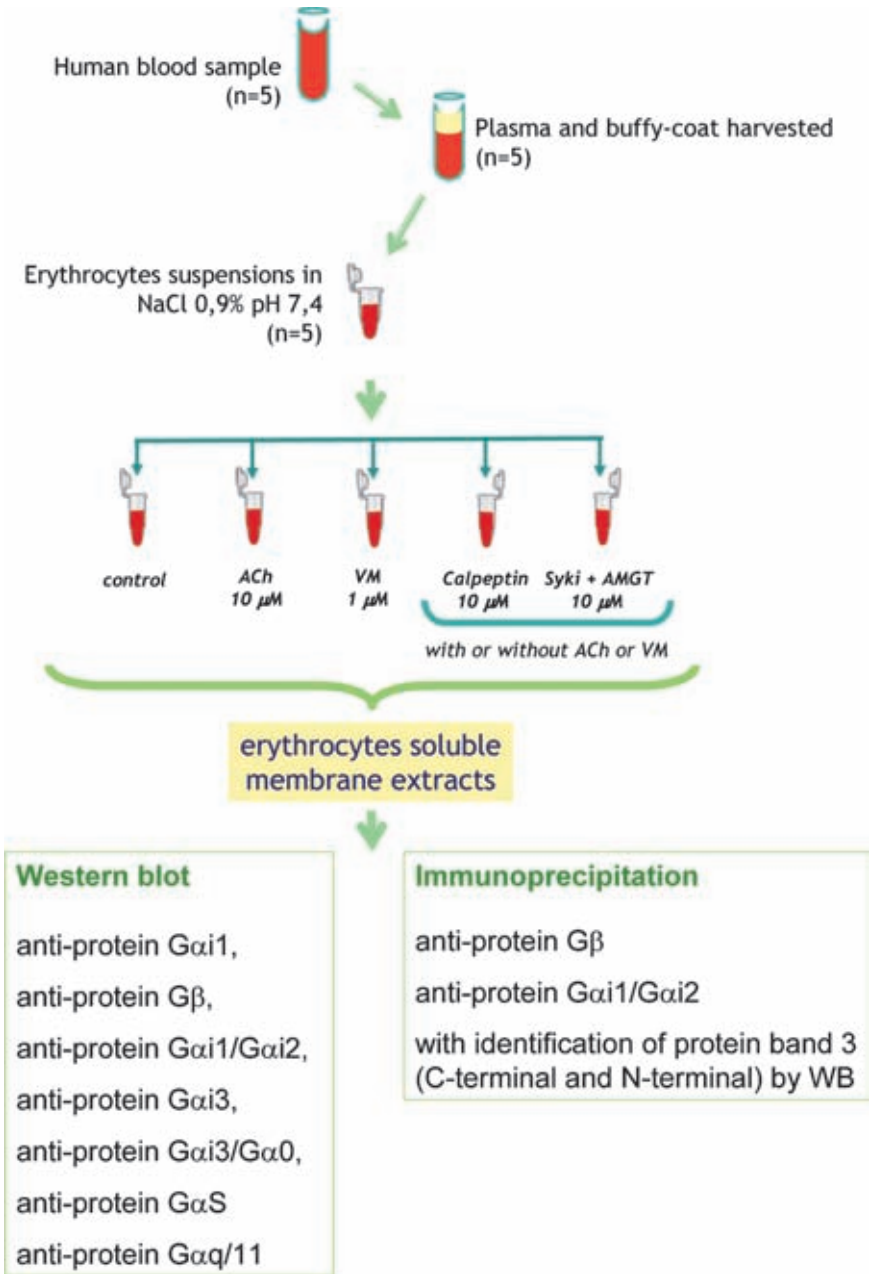


Fig. 1 – **(A)** Presentation of the hypothesis for acetylcholinesterase role on signal transduction mechanism in response to action of ACh or VM on NO production (and its metabolites) in human erythrocytes suspensions is mediated by transnitrosilation processes between phosphorylated / dephosphorylated band 3 when PTK or PTP enzymatic activity are inhibited. This process could be dependent of a G protein (α,β,γ) (Carvalho F.A. et al (2004).
(B) Receptors coupled to heterotrimeric GTP-binding proteins (G proteins) are integral transmembrane proteins that transduce extracellular signals to the cell interior. Receptor occupation promotes interaction between the receptor and the G protein on the interior surface of the membrane. This induces an exchange of GDP for GTP on the G protein a subunit and dissociation of the a subunit from the bg heterodimer. Depending on its isoform, the GTP-a subunit complex mediates intracellular signaling either indirectly by acting on effector molecules such as adenylyl cyclase (AC) or phospholipase C (PLC), or directly by regulating ion channel or kinase function.[2]

related to the transnitrosilation process between phosphorylated / dephosphorylated band 3 and SNOHb could be associated to an unidentified mechanism mediated by formation of AChE-ACh (more active) or AChE-VM (less active) complexes, when PTK or PTP enzymatic activity is inhibited. When we proposed this signal transduction mechanism we thought that this process could also be dependent of a G protein. So the aim of this study was to identify the G protein form that could be linked to the protein band 3 and to know what protein G sub-units (α,β,γ) are related to the activation or inhibition of acetylcholinesterase and band 3 phosphorylation degree states.

EXPERIMENTAL DESIGN



RESULTS

We could observe from Western blotting analysis (vd. Fig. 2) that protein Gβ and protein Gαi1/Gαi2 could be linked to the erythrocyte protein band 3. All the others protein G isoforms tested are identified on erythrocytes membrane extracts but there is no evidence that could be linked to protein band 3. The immunoprecipitation analysis between the protein Gβ or protein Gαi1/Gαi2 and protein band 3 confirm the previous Western blotting results (vd. Fig. 3 and 4)

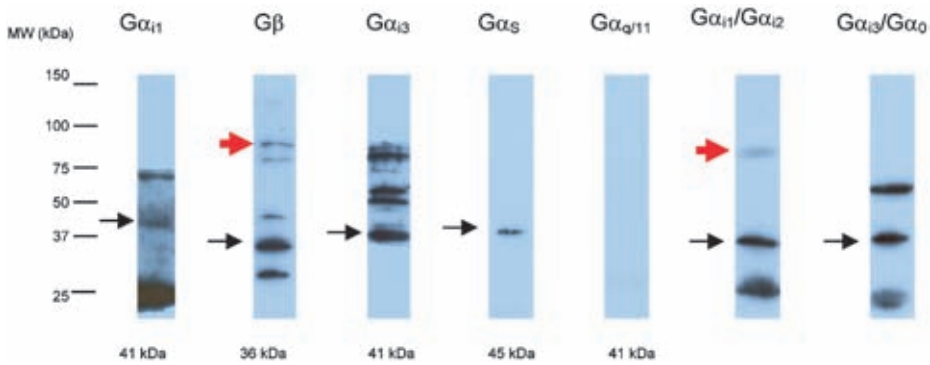


Fig. 2 – Detection of protein Gαi1, protein Gβ, protein Gαi1/Gαi2, protein Gαi3, protein Gαi3/Gα0, protein GαS and protein Gαq/11 on erythrocyte membrane soluble extracts (control) by immunoblotting.

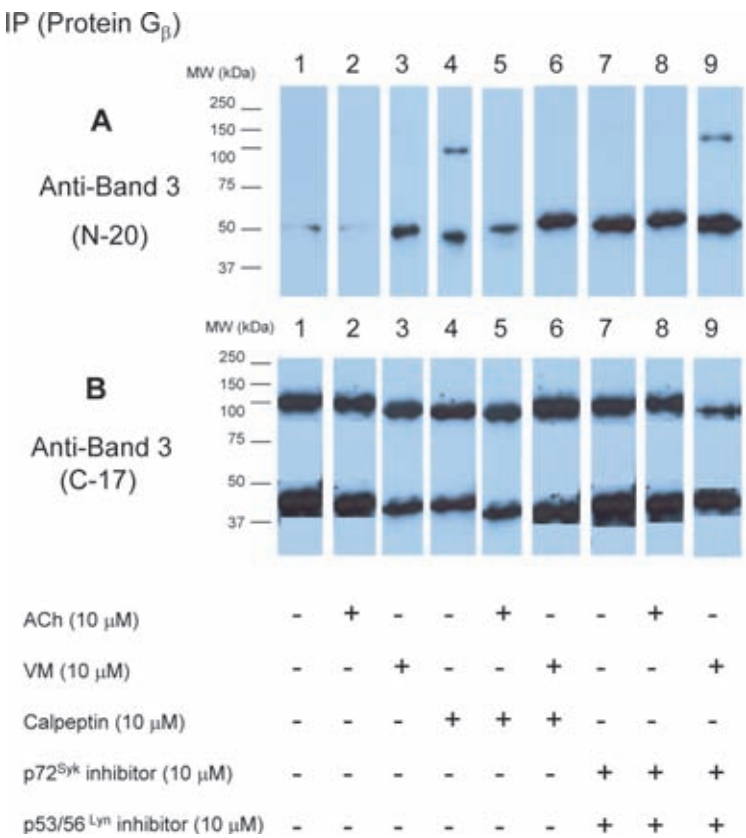


Fig. 3 – Immunoprecipitation of protein Gβ on erythrocyte membrane soluble extracts previously incubated with control (lane 1), ACh 10 μM (lane 2), VM 10 μM (lane 3), p72syk inhibitor 10 μM (lane 4) with ACh (lane 5) or VM (lane 6) and p53/56lyn inhibitor 10 μM (lane 7) with ACh (lane 8) or VM (lane 9). The immunoprecipitate was electrophoresed and immunoblotted with anti-band 3 antibodies for C-terminal and N-terminal.

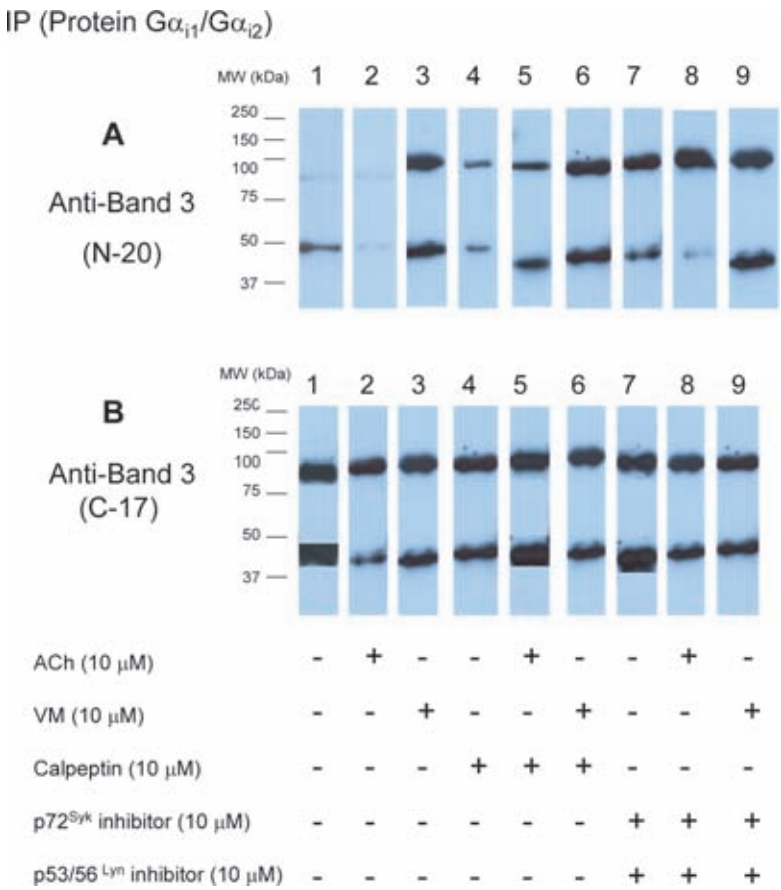


Fig. 4 – Immunoprecipitation of protein Gαi1/Gαi2 on erythrocyte membrane soluble extracts previously incubated with control (lane 1), ACh 10 μM (lane 2), VM 10 μM (lane 3), p72syk inhibitor 10 μM (lane 4) with ACh (lane 5) or VM (lane 6) and p53/56lyn inhibitor 10 μM (lane 7) with ACh (lane 8) or VM (lane 9). The immunoprecipitate was after submitted to Western blotting with anti-band 3 antibodies for C-terminal and N-terminal.

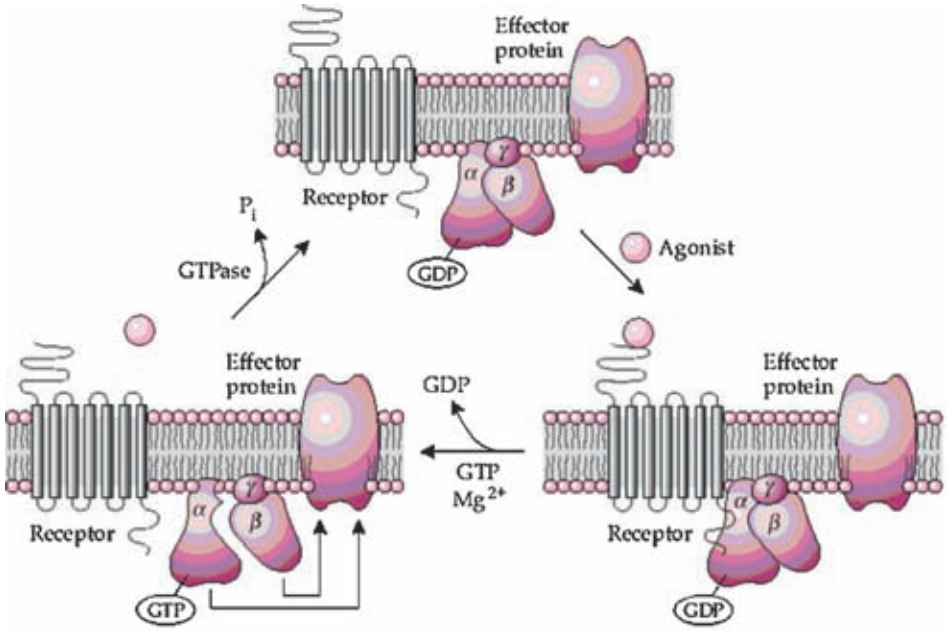
CONCLUSIONS

The results of this work allow us to

- A possible interaction between protein Gαi1/Gαi2 and/or protein Gβ and protein band 3 on erythrocytes membrane
- Band 3 C-terminal: both protein Gαi1/Gαi2 and protein Gβ may be bonded for immunoprecipitation
- Band 3 N-terminal: protein Gαi1/Gαi2 may be bonded for immunoprecipitation; Gβ may be bonded only when band 3 is phosphorylated and when PTK inhibitors are incubated with velnacrine
- Stimulation with ACh (with or without PTK inhibitors) – increase of the linkage Gαi1/Gαi2 – band 3 (C-terminal) expression
- The observed two different conformational G protein sub-units stages seems to be related with the phosphorylation band 3 protein

From these results we proposed that a possible G protein sub-unit conformational mechanism that could be related with our findings could be the one indicated here.[3]

The heterotrimeric G protein Gi, participates in the ATP release from erythrocytes[4]. The expression of G_{i2} is reduced in the erythrocyte membranes of humans with type 2 diabetes[5]. It was also verified a decreased of G proteins (Gαi, Gαo and Gβ) in hypertensive subjects[6]. All together these results suggests that this defect in erythrocyte physiology could lead to a reduced stimulus for endogenous NO synthesis in the microvasculature,



In summary, our results allows us to confirm that G_i is a necessary component of the signal transduction pathway that seems to be linked to the protein band 3 phosphorylation degree states, and related to the activation or inhibition of acetylcholinesterase complex and supports our proposed erythrocyte NO translocation mechanism.

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OXIDATIVE STRESS, ENDOTHELIAL DYSFUNCTION AND VASCULAR DISEASE ON OBESITY AND PENILE ERECTILE DYSFUNCTION

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ABSTRACT

Different cardiovascular risks factors are associated with fewer endothelial nitric oxide bioavailability and endothelial dysfunction. Obesity is one of the Occidental diseases that have an improved of cardiovascular morbility and mortality. Obesity and penile erectile dysfunction are causes of an oxidative stress induced by oxygen reactive species and free radicals production that leads to endothelial dysfunction and vascular disease. Nitric oxide is an important vasodilator messenger interfering in these diseases. So we proposed to study possible changes on biochemical and hemorheological parameters on Obesity Women (n=24) and penile erectile dysfunction (n=18) comparing with the levels achieved of healthy persons (control, n=10). We quantified nitric oxide levels by an amperometric method, acetylcholinesterase activity, blood viscosity, plasma viscosity, erythrocyte aggregation (5 and 10 s), erythrocyte deformability and fibrinogen concentration. The obtained NO concentration values were: 1.66 ± 0.44 nM (control), 2.1 ± 0.43 nM (obesity, P = 0.027) and 1.90 ± 0.43 nM (erectile dysfunction). Fibrinogen concentration significant increase in both pathologies studied (292.7 ± 54.3 mg/dL for obesity (P = 0.0004) and 289.6 ± 93.0 mg/dL for erectile dysfunction (P = 0.033) vs. 206.7 ± 23.8 mg/dL for control group). Erythrocyte aggregation levels at 10 s are significantly augmented in both pathologies (P = 0.012 for obesity and P = 0.05) for erectile dysfunction) but only obesity erythrocyte aggregation levels significant increase at 5 s (P = 0,002). Also, blood viscosity of erectile dysfunction group significant increase the ones of control group (3.9 ± 0.4 mPa.s vs 3.3 ± 0.4 mPa.s (control), P = 0.003). All other biochemical and hemorheological parameters do not showed statistically significant variation. In conclusion, both diseases produce biochemical and hemorheological disorders that could be markers of future therapeutic action on vascular and microcirculatory dysfunction, by controlling tissue oxygenation.

INTRODUCTION

Obesity is associated with abnormal endothelial function (Arcaro G et al 1999) It is often inferred that the reduction in endothelial function is the result of a decrease in nitric oxide (NO). Decreased NO in obesity may be related to an increase in oxidative stress (Lee KU 2001) or may result from proinflammatory cytokines. A decrease in the function of NO would result in vasoconstriction and an increase in vascular resistance that may predispose to cardiovascular disease risk factors such as hypertension.

Vascular disease is by far the most common cause of erectile dysfuncion. Formerly dismissed as a psychological condition, erectile dysfuncion has now assumed center stage as a readily treatable disorder and a powerful risk-marker for cardiovascular disease. (Shabsigh R et al, 2003, Montorsi P et al, 2004).

The current knowledge of the pathogenesis of vasculogenic erectile dysfuncion, with specific reference to abnormalities in the biology of nitric oxide (NO) and the relationship between and the clinical management of erectile dysfuncion and cardiovascular disease. Smooth muscle cell relaxation is achieved through the cyclic GMP and cyclic AMP pathways, both of which are modulated by various chemomediators. Penile tumescence and erection is critically reliant on the release of NO by both cavernosal nerve terminals and endothelial cells (Maas R et al, 2002; Saenz de Tejada I. et al, 2004).

The role of the endothelial cells in the maintenance of penile erection underscores the close association of erectile dysfuncion with endothelial dysfunction in the peripheral circulation, and with the presence of cardio vascular risk factors and coronary artery disease (Solomon H. et al, 2003; Bortolotti A. et al 1994).

Endothelial dysfunction due to an abnormality in the release and/or action of NO is characterized by vasoconstriction, coagulation, increased leucocyte adhesion and stimulation of smooth muscle cell growth. Several traditional cardiovascular risk factors, such as aging, smoking, hypertension, dyslipidemia and diabetes, and some less- traditional risk factors, including inflammation, hypoxia, oxidative stress and homocysteinemia, are related to endothelial dysfunction (Celermajer DS et al, 1994, Brunner H et al, 2005).

So, endothelial and erectile dysfunctions are intimately associated; both disorders arise from disturbance in the release and action of nitric oxide from endothelial cells. Erectile and endothelial dysfunction can be readily detected clinically and are associated with a wide spectrum of cardiovascular risk factors, and are also independently predictive of cardiovascular events.

As so, we proposed to study the biochemical and hemorheological parameters that are possible related to oxidative stress and endothelial dysfunction of the two different vascular diseases such as, obesity (women, n = 24) and penile erectile dysfunction (n = 18) and compare with control levels from healthy persons (n = 10).

EXPERIMENTAL DESIGN

Venous blood samples were collected from healthy humans (n = 10) and woman patients with obesity (n = 24) and penile erectile dysfunction (n = 18). Erythrocyte suspensions from healthy humans were performed with addition of sodium chloride 0,9%, pH 7,4, to reconstitute the initial hematocrit (Ht, 45 %). Biochemical and hemorheological parameters were measured such as, amperometric NO quantification, fibrinogen concentration, acetylcholinesterase activity, erythrocyte aggregation, erythrocyte deformability and plasma and blood viscosity.

Determination of erythrocyte deformability – erythrocyte was determined using the Rheodyn SSD laser difractometer, following the guidelines of the International Committee for Standardization in Haematology. The Rheodyn

SSD difractometer determines erythrocyte deformability by simulating the shear stresses exerted by the blood flow and vascular walls on the erythrocytes. Erythrocytes are suspended in a viscous medium and placed between a rotating optical disk and a stationary disk, where they are going to be subjected to well defined shear stress, which forces the erythrocytes to deform to ellipsoids and align with the fluid shear stress.

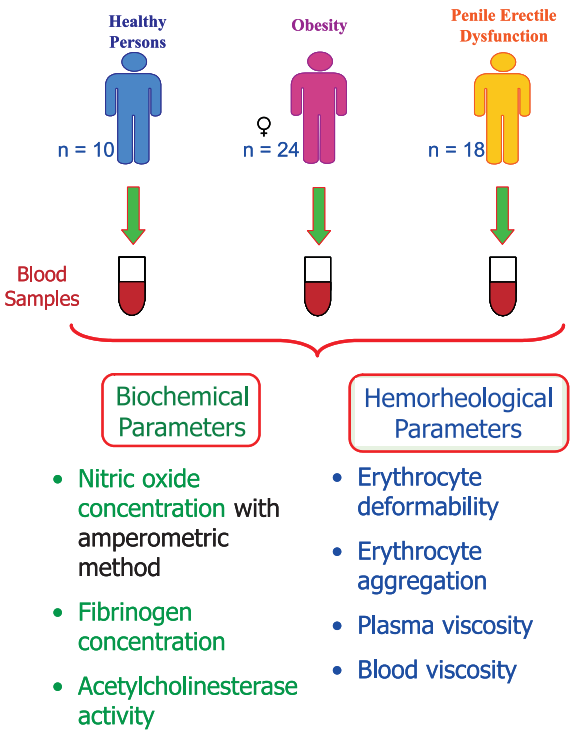

Fig. 1 – Experimental design.

Determination of erythrocyte aggregation – was determined using the MA1 aggregometer. The MA1 aggregometer consists of a rotative cone-plate aggregometer that disperses the sample by high shear stress (600/s), and a photometer that determines the extent of aggregation. The aggregation will be determined by to ways: (a) in stasis during 10 seconds, and (b) at shear rate of 4/s during 5 seconds, after dispersion of the blood sample.

Measurement of nitric oxide concentration – Nitric oxide was determined by an amperometric method using an amiNO-IV sensor (Carvalho FA et al, 2004). The erythrocytes suspensions (hematocrit = 0.05%) will be stimulated with acetylcholine 10 μM and erythrocytes nitric oxide mobilization monitorized.

Measurement of erythrocyte acetylcholinesterase activity – AChE activity was measured using a spectrophotometer by the colorimetric method of Ellman et al and adapted to erythrocytes by Kaplan et al.

Measurement of plasma and blood viscosity – Plasma viscosity of the samples will be measured using a capillary viscometer according to the Harkness method. Whole-blood will be measured with a rotation digital viscosimeter.

Measurement of fibrinogen concentration – The fibrinogen concentration will be measured in a Fibrintimer, using a modification of the Clauss method.

RESULTS AND CONCLUSIONS

From Fig.2 (A) we observed that there is a significant increase of NO concentration on obesity patients (P = 0.027). Erythrocytes of penile erectile dysfunction patients mobilized more the nitric oxide then the control group but this result is not statistically significant.

We observed that fibrinogen levels are statistically increased in both pathologies (P = 0.0004 in obesity, P = 0.033 in erectile dysfunction). Fibrinogen, who basic physiological function in hemostasis is the formation of a fibrin network, is a major determinant of whole blood viscosity and it is an inflammatory marker classified as an acute phase protein (vd. Fig.2 (B)).

Acetylcholinesterase is an erythrocyte enzyme membrane that is stated as a marker of membrane integrity. As we could observed from Fig.2(C),

obesity and erectile dysfunction AChE activity levels had no significant changes related to control patients, so we may conclude that erythrocyte membrane remains its integrity despite the endothelial dysfunction and vascular disorders already knew on two pathologies.

From the study of hemorheological parameters (vd. Fig. 3) we could see that blood viscosity

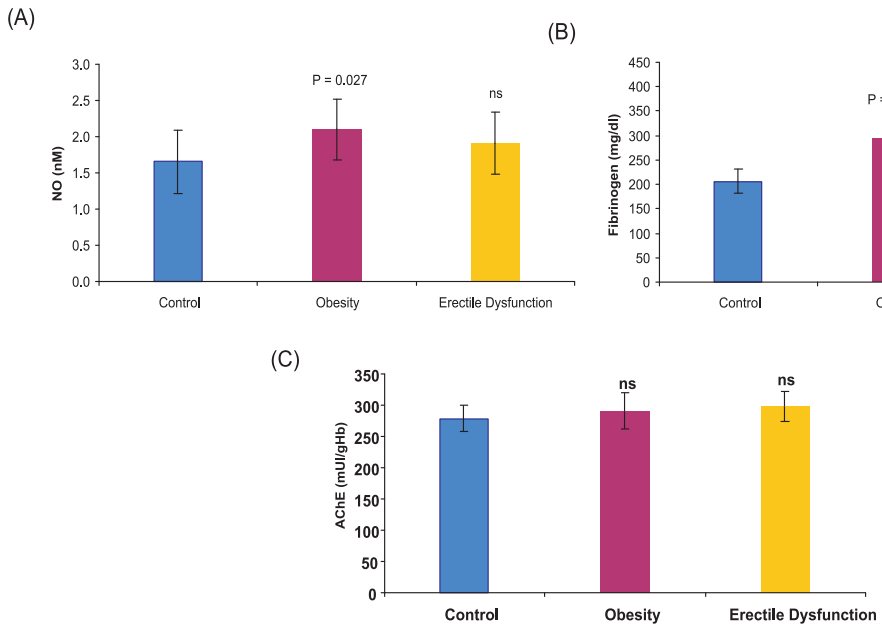

Fig. 2 – Biochemical parameters: nitric oxide concentration (A), fibrinogen concentration (B) and acetylcholinesterase activity (C). Values are from control (healthy persons) and from patients with obesity and penile erectile dysfunction. Values are in mean ± SD

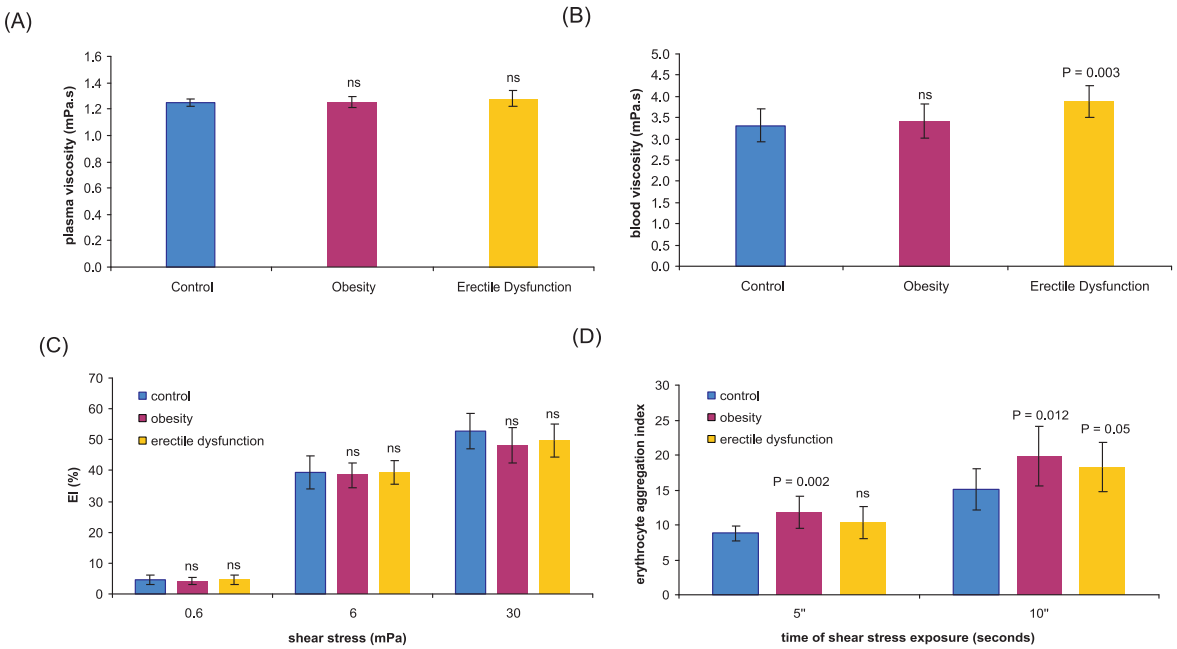

Fig. 3 – Hemorheological parameters: plasma viscosity (A), blood viscosity (B), erythrocyte deformability (C) and erythrocyte aggregation (D). Values are from control (healthy persons) and from patients with obesity and penile erectile dysfunction. Values are in mean ± SD.

significant increase on penile erectile dysfunction (P = 0.003). Plasma viscosity values and erythrocyte deformability had no significant changes on both pathologies. At 10 seconds of shear stress erythrocytes exposure, we observed a statistically significant increase of erythrocyte aggregation of both pathologies (obesity P = 0.012 and erectile dysfunction P = 0.05). This increase was also observed at 5 seconds of shear stress erythrocytes exposure for obesity patients (P = 0.002, vd. Fig 3(D)).

We could see from these results that obesity and penile erectile dysfunction leads to different biochemical and hemorheological disorders that could be markers of future therapeutic action on vascular and microcirculatory dysfunction, by controlling tissue oxygenation. Both pathologies are causes of an oxidative stress induced by oxygen reactive species and free radicals production that leads to endothelial dysfunction, inflammation and vascular disease.

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KDR – A NUCLEAR SIGNALLING PROTEIN

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ABSTRACT

Activation of vascular endothelial growth factor receptor-2 (KDR) on endothelial cells (EC) plays a crucial role in both physiological and pathological angiogenesis. However, the molecular mechanisms that regulate KDR expression and turnover remain unclear.

Our results show that on Human Umbilical Vein EC (HUVEC), VEGF stimulation induces KDR and VEGF nuclear accumulation. Furthermore, the KDR nuclear accumulation was inhibited using an inhibitor of KDR phosphorylation (KDRi) or a neutralizing antibody against FLT-1(6.12Ab). Moreover, our results demonstrate the importance of the cross-talk between the 2 VEGF receptors in KDR internalization. Additionally, we demonstrate that in vitro wounding of EC monolayers leads to a rapid and transient internalization of VEGF + KDR to the nucleus, which is essential for monolayer recovery. Notably, FLT-1 blockade impedes VEGF and KDR activation and internalization, blocking endothelial monolayer recovery. Also, we established 3 KDR deletion mutants; in these, VEGF-induced KDR internalization was impaired, demonstrating that this process requires activation (phosphorylation) of the receptor. So, a variety of point mutants of KDR fused to GFP are being constructed in order to examine which tyrosine residues are involved in KDR internalization.

INTRODUCTION

Angiogenesis is a fundamental developmental and adult physiological process of blood vessels formation from pre-existent ones. Several pathological conditions such as, tumor progression and chronic wounds are characterized by a de-regulation of the angiogenic balance which requires the coordinated action of a variety of growth factors and cell adhesions molecules[1].

Vascular endothelial cells (ECs) that line the inside of the blood vessels as a monolayer have long been considered to be a key component for angiogenesis, acting in response to local and systemic stimuli by resisting apoptosis, proliferating and migrating[2]. The molecular pathways that mediate these responses have been under intense scrutiny. The Vascular Endothelial Growth Factor (VEGF) is considered to be the major regulator for angiogenesis. VEGF is produced by the majority of cells in the body, in response to hypoxia or inflammation, but acts mainly on ECs, promoting their survival, proliferation and migration in situations such as the ones described above. The biological effects of VEGF are mediated, in ECs, via two specific cell surface-expressed tyrosine kinase receptors, VEGFR-1 (also known as FLT-1) and VEGFR-2 (also known as KDR). Although the affinity for VEGF is higher for Flt-1 than for KDR, it is the activation of the later that is responsible for conveying the VEGF-mediated effects on ECs. VEGF binds to the extracellular domain of KDR and induces the dimerization and the autophosphorylation of specific intracellular tyrosine residues; consequently several signalling pathways are activated, leading to specific cellular functions, such as proliferation, migration, permeability and survival[3].

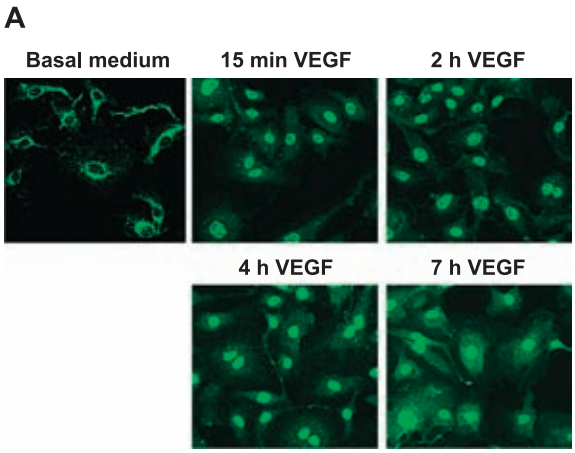
Our recently published results[4] show that on Human Umbilical Vein EC (HUVEC), VEGF stimulation induces KDR and VEGF nuclear accumulation. This previous unrecognized cellular localization of KDR may reveal its important role as a nuclear signalling protein in ECs and subsequently, as a new therapeutic target. Furthermore, the KDR nuclear accumulation was inhibited using a specific intracellular inhibitor of KDR phosphorylation (KDRi), suggesting that VEGF induces KDR phosphorylation, thereby allowing internalization to occur[4]. Also, VEGF treatment in the presence of a neutralizing antibody against FLT-1 blocked KDR nuclear internalization. The decrease in KDR nuclear internalization was accompanied by an accumulation of membrane KDR. These results suggest that VEGF binds FLT-1 and the crosstalk between the 2 VEGF receptors results in KDR activation and nuclear internalization[4].

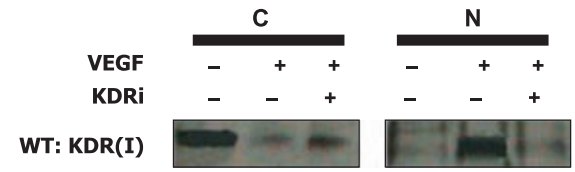
These previous results suggested that KDR phosphorylation is crucial for KDR nuclear internalization. Several autophosphorylation sites have been identified in the intracellular region of KDR: Tyr 951 and Tyr 996 in the kinase insert; Tyr 1054 and Tyr 1059 in the kinase domain and Tyr1175, Tyr1212, Tyr1214 and Tyr1305 in the C-terminal tail. According to this, we established 3 KDR deletion mutants in 3 tyrosine residues; in these, VEGF-induced KDR internalization was impaired, demonstrating that this process requires activation (phosphorylation) of the receptor[4].

Additionally, we demonstrated that in vitro wounding of ECs monolayers leads to a rapid and transient internalization of VEGF + KDR to the nucleus, which is essential for monolayer recovery. Notably, FLT-1 blockade impedes VEGF and KDR activation and internalization, blocking endothelial monolayer recovery[4].

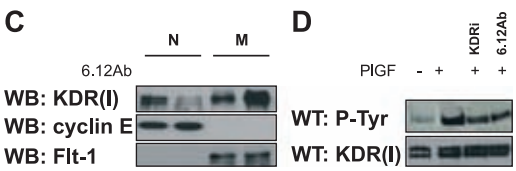
RESULTS AND DISCUSSION

Both Western Blot (WB) analysis and immunohistochemical studies showed that VEGF induced nuclear KDR expression on endothelial cells (EC) (A and B). A KDR-specific tyrosine kinase inhibitor (KDRi-70nM) blocked VEGF-induced KDR nuclear expression while decreasing its cytosolic levels (B).

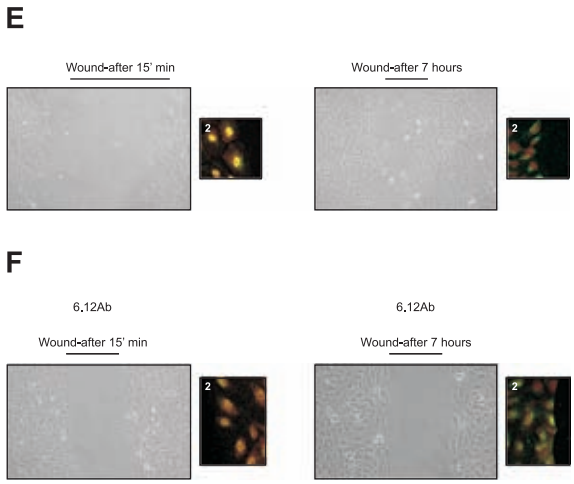




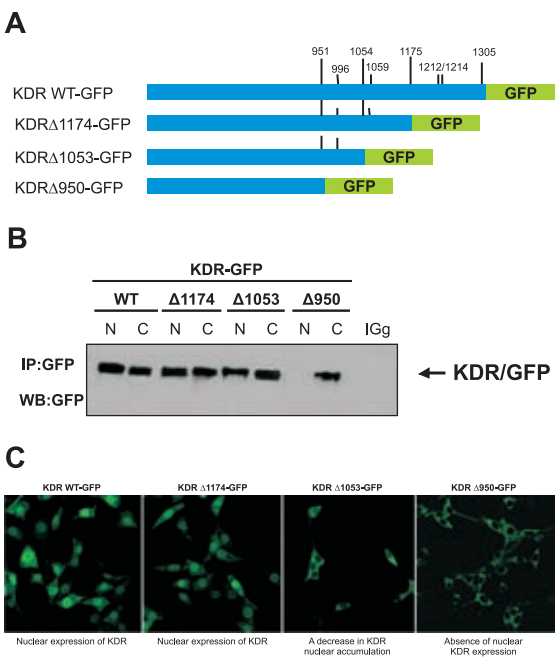
The same effect is obtained with VEGF and a neutralizing antibody against FLT-1 (clone 6.12Ab) (C). Activation of FLT-1, through the use of PlGF (10ng/ml) results in KDR phosphorylation by a mechanism inhibited by KDRi or 6.12Ab (D).



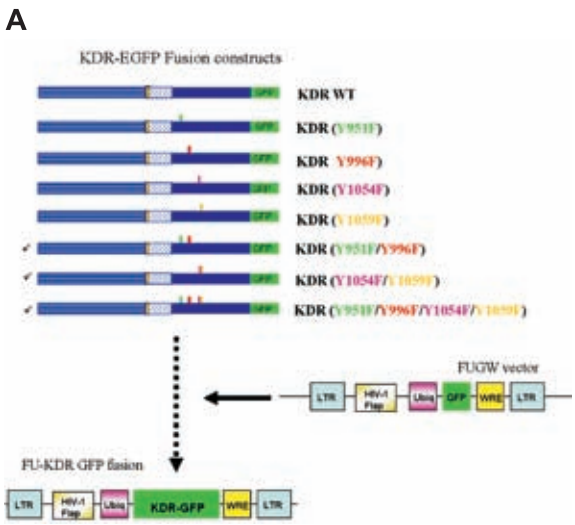
Using an in vitro wound healing model, where confluent EC monolayers suffer mechanical wounding, the results show that KDR and VEGF accumulate intracellularly in EC facing the wound, proceeding monolayer recovery (E). Treatment of the EC monolayer with 6.12Ab, prior to wounding, delayed monolayer recovery and by immunofluorescence analysis we can see that no KDR and VEGF internalization was observed in cells facing the wound (F).



Several auto-phosphorylation sites have been identified in the intracellular region of KDR: Tyr951 and Tyr996 in the kinase insert; Tyr1054 and Tyr1059 in the kinase domain and Tyr1175, Tyr1212, Tyr1214 and Tyr 1305 in the C-terminal tail. In order to reveal the role of these different tyrosines residues in the process of KDR internalization, we established 3 KDR deletion mutants for the intracellular region (A). Wild-type and KDR mutants were transfected in NIH3T3 cells. KDR expression was analysed by immunoprecipitation (IP) (B) and confocal microscopy (C).

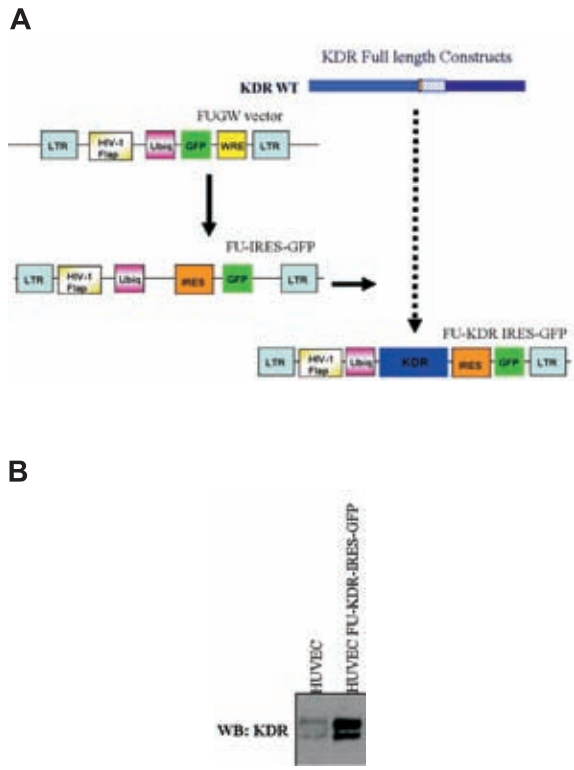


According to deletion mutants results, we constructed a variety of point mutants of KDR fused to GFP by mutating tyrosine to phenylalanine (A). The different constructs are being cloned in a lentiviral vector (A) in order to infect EC and to examine which tyrosine residues are involved in KDR internalization.



KDR WT was also cloned in a bicistronic lentiviral vector – FU-IRES GFP (A) and HUVECs were infected. The KDR overexpression was confirmed by WB (B) using protein extracts.

Once we confirmed that HUVEC are overexpressing nuclear KDR, we will investigate how this nuclear overexpression regulates the DNA binding activity of transcription factors (Tfs) important in cell proliferation, migration and survival.



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VASCULATURE ACTIVATION AND TUMOR RE-GROWTH AFTER RADIOTHERAPY

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ABSTRACT

Since tumor growth is angiogenesis-dependent, detailed molecular and cellular studies are thus needed to understand the parameters implicated in the interactions between the tumor and vasculature compartment with the objective of improving therapeutic strategies, not only for cancer treatment but also for preventing recurrence. In this context, the possible pro-angiogenic effects of radiotherapy on tumor vasculature, activating or promoting resistance on the endothelial cells (ECs), has been poorly characterized.

In the present study, while investigating the pro-angiogenic effects of low doses of irradiation in the vasculature, our results suggest that low doses of irradiation below 1.0 Gy are able to induce a pro-angiogenic response to wound healing without affecting cell survival or proliferation.

In order to confirm a differential response of ECs to low doses of radiation at molecular level, lung human microvascular ECs (HMVEC-L) were irradiated at 0.5 Gy and the level of tyrosine phosphorylation was analysed. Our results show that low doses of ionizing radiation are responsible for an increase of the tyrosine phosphorylation level. In the future, we propose to identify the mechanisms whereby low doses of irradiation induce a pro-angiogenic response in the vasculature in order to know in which way this process may be involved in tumor re-growth.

INTRODUCTION

Angiogenesis is the development of new microvessels from pre-existing vasculature and it is a requisite for many physiological and pathological processes such as wound healing and tumor growth. In 1971, Folkman proposed that tumor growth and metastasis are angiogenic dependent, and hence, blocking angiogenesis would be an effective strategy to arrest tumor growth and treat human cancer[1]. Several regulators of angiogenesis were identified and some of these represent therapeutic targets. One of the most important pro-angiogenic factors is the Vascular Endothelial Growth Factor (VEGF) that promotes endothelial cell proliferation, angiogenesis, and tumor growth, and maintains the elevated vascular permeability of tumor vessels[2]. In addition, several studies demonstrate that VEGF may play a crucial role in tumor recurrence. It was suggested that VEGF release following surgical procedures stimulates a local angiogenic response that might contribute towards the re-growth of the tumor[3-6]. On the other hand, irradiation was shown to lead to VEGF production by the tumor, which in turn may induce anti-apoptotic pathways and promote tumor re-growth[7-14]. Moreover, several studies demonstrated that anti-angiogenic approaches can enhance radiation-induced tumor growth inhibition by different mechanisms that include an increase in tumor oxygenation, a decrease in vascular density and radiosensitization of endothelial cells (ECs)[7-8]. Interestingly, another study demonstrated that irradiation doses of 2 and 6 Gy induced EC migration and sprouting through up-regulation of the endothelial pro-angiogenic NO pathway[15]. This data is not contradictory with the concept that the cytotoxic effects of high doses of radiation on ECs contribute to anti-tumoral treatment, as previously reported[16], but suggest that a proportion of ECs did survive the radiation stress and this selected population may undergo the angiogenic phenotypic shift.

The concept that irradiation itself may generate collateral effects conferring resistance to ECs brings the need to new approaches in order to avoid tumor re-growth after irradiation.

In the context of tumor cells per se it was described[17] that radiation activates signalling pathways which are involved in cell survival; inhibition of these pathways decreases tumor growth and survival after irradiation. Indeed, in most of the studies, the final read-out of the co-therapy focused on the effects on tumor growth, but the impact of irradiation on the vasculature was not well addressed.

This issue becomes extremely important if we think about the radiotherapy procedure, since the total dose is fractionated in order to give time to normal cells (including endothelial cells) to recover. Typically the dose is given at 1.8-2.0 Gy per day for adults, or 1.5-1.7 Gy per day for children[18]. Moreover, in order to spare normal tissues, shaped radiation beams are aimed from several angles of exposure to intersect at the tumor, providing a much larger absorbed dose there than in the surrounding healthy tissues[18]. So, the amount of radiation received by tumor-surrounding vasculature is generally much lower than the 1.8-2.0 Gy defined dose.

This leads us to the following questions: what is the effect of those low doses of radiation below 2.0 Gy on the vascular cells? Are these low doses unable to trigger any vascular response or, on the contrary, they activate ECs promoting a pro-angiogenic response?

If this is the case, how can this process be involved in tumor re-growth and how may these low doses of radiation be used as a therapy process in pathologies associated with deficient angiogenesis?

In this project, we've proposed to define a pro-angiogenic (activating) dose of ionizing radiation, and subsequently characterize ECs in response to this pro-angiogenic irradiation. The effects of irradiation are being studied on human lung microvasculature ECs (HMVEC-L) in order to identify the targets that could play a role in angiogenesis and tumor re-growth.

RESULTS AND DISCUSSION

HMVEC-L received single doses of radiation, performed at room temperature using a linear accelerator X-rays photon beam, operating at 6 MV (Varian Clinac 2100 CD) with a dose rate of 300 MU/min. To assure the accurate and homogeneous distribution of the chosen radiation dose, an acrylic phantom box was constructed and a careful dosimetry protocol was planned by the treatment planning system through mathematical models.

Radiation induces endothelial cell migration at 0.5 and 0.8 Gy – Directed cell migration is a key component of the angiogenic process. In order to analyze the effect of low doses of radiation in the ECs migration, HMVEC-L were submitted to different ionizing radiation doses and an in vitro wound healing assay was performed, where confluent monolayers suffer mechanical scratch injury.

Migration assays suggest that 0.5 and 0.8 Gy doses are able to stimulate the ability of HMVEC-L to migrate toward the center of the wound (Fig.1), while cells irradiated at 1.0 Gy present a recovery rate similar to the control condition. Note that at 12 hours recovery, the cells irradiated at 0.5 Gy present the scratched area entirely repopulated.

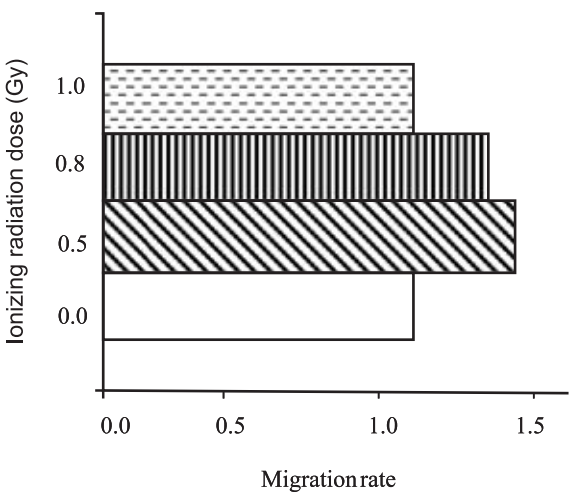

Fig. 1 – Low doses of radiation below 1.0 Gy are able to induce ECs migration.

HMVEC-L survival is not affected by low doses of radiation below 5.0 Gy – HMVEC-L survival was determined at 24, 48 and 72 hours after irradiation. Cells were double stained with Annexin V and Propidium Iodide (PI), and the percentage of apoptotic cells was determined by flow cytometry (FACS) (Fig.2). Cells cultured in deprivation conditions (Dep) were used as control. Values are given as the percentage of viable cells (Annexin V negative cells). No effects on cell viability are verified until 5.0 Gy.

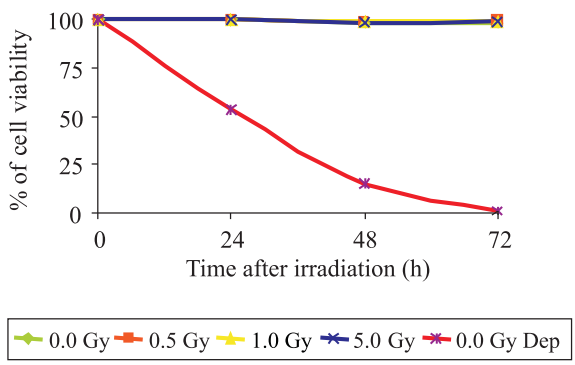

Fig. 2 – Percentage of HMVEC-L viability. Note that cell survival is not affected by low doses of radiation until 5.0 Gy in normal culture conditions. Cells cultured in the absence of growth factors (Dep) were used as a positive death control.

Proliferation is not affected by low doses of irradiation below 1.0 Gy – To assess the effects of increasing radiation doses on ECs proliferation, HMVEC-L were plated at equal densities; after 12 hours, the cells were or not irradiated. After 72 hours the cells were counted using a hemacytometer. As shown in Figure 3, radiation doses below 1.0 Gy do not cause any alteration on HMVEC-L proliferation; however, doses higher than 1.0 Gy are responsible for a significant decrease in the proliferation rate.

Cell cycle profile, obtained by PI / FACS analysis, supports the previous results (data not shown).

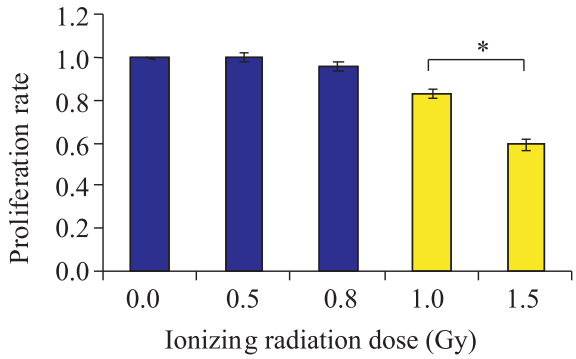

Fig. 3 – HMVEC-L proliferation and cell cycle profile 72 hours post-radiation. Hemacytometer cell count shows that proliferation rate is not affected by low doses of ionizing radiation below 1.0 Gy; higher doses are responsible for a significant decrease in the cell number (*$p<0.05$ with respect to the 0 Gy)

Low doses of ionizing radiation are responsible for an increase in the tyrosine phosphorylation level – In order to confirm a differential response of HMVEC-L to low doses of radiation at molecular level, cells in normal culture conditions were irradiated at 0.5 or 0,8 Gy and cultured for 5 minutes. The level of tyrosine phosphorylation was analyzed by flow cytometry (Fig. 4) and confirmed by Western Blot, using protein extracts (data not shown). An increase of the tyrosine residues phosphorylation level was detected 5 minutes after radiation.

Our results suggest that, low doses of ionizing radiation below 1.0 Gy are able to induce a pro-angiogenic response to wound healing without affecting cell survival and proliferation. Furthermore, our results show that low doses of ionizing radiation are responsible for an increase of the tyrosine phosphorylation level, reflecting an enhanced intracellular activity.

In order to confirm the ability of low doses of radiation to activate ECs, inducing a pro-angiogenic response, we're also preparing an in vitro system of human microvascular sprout angiogenesis, where we'll analyze the capacity of ECs to form capillary-like sprouts and branching in response to irradiation.

In the future, we'll genetically characterize the pro-angiogenic response of ECs to radiation using cDNA microarray analysis, in an attempt to reveal new targets involved in vasculature activation. The results will be confirmed and validated, and the role of the more relevant target genes in the pro-angiogenic response will be evaluated. We will also perform some in vivo studies in order to validate our in vitro results, and understand the role of the microenvironment in the ECs responsiveness.

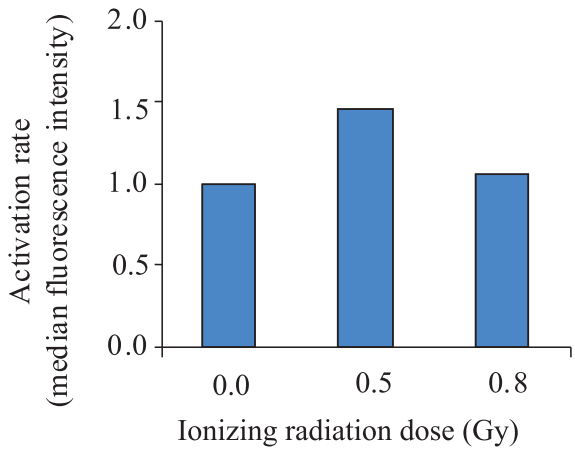

Fig. 4 – Flow cytometry analysis show an increase tyrosine phosphorylation 5 minutes post- radiation at 0.5 Gy. Results are shown in median fluorescence intensity.

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THE MODULATION OF CYCLIC NUCLEOTIDE LEVELS AND PKC ACTIVITY BY ACETYLCHOLINESTERASE EFFECTORS IN HUMAN ERYTHROCYTES

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ABSTRACT

Background. The non-neuronal cholinergic system, modulated by acetylcholine, is widely distributed in biological systems, contributing to organ and blood homeostasis. In human erythrocytes, acetylcholine has been proved to modulate several crucial phenomena as of hemorheology parameters and nitric oxide mobilization, with involvement of membrane-bound acetylcholinesterase. The influence of this dual system on signalling transduction pathways involving cAMP/cGMP and protein kinase C (PKC), still unknown, was studied here. **Methods.** From blood samples of 15 healthy donors, erythrocyte suspensions (ES) were prepared and incubated with acetylcholinesterase substrate (acetylcholine) and inhibitor (velnacrine maleate), and with adenylate cyclase / guanylate cyclase inhibitors. The levels of cAMP/cGMP and PKC activity were determined afterwards by using enzyme immunoassay kits and a spectrofluorimetric method employing a fim-1 diacetate specific fluorescent probe, respectively. **Results.** The presence of the acetylcholine in ES increases cGMP and decreases cAMP, at variance, velnacrine enhances both messengers. Inhibited guanylate cyclase led to lower cGMP but higher cAMP with both effectors. By turn, inhibited adenylate cyclase let to lower cAMP with both effectors and decreased cGMP with velnacrine. Regarding to the PKC activity we observed a significant decrease when guanylate cyclase inhibitor is present and no significant changes occur in presence of adenylate cyclase inhibitor. The simultaneously erythrocyte incubation with adenylate cyclase inhibitor and acetylcholinesterase effectors induced an increase of PKC activity. The same was verified when guanylate cyclase inhibitor was present instead of adenylate cyclase inhibitor. **Conclusion.** Acetylcholinesterase is involved in the signalling cascades involving second messengers, and a cross-talk mechanism concerning PDE III blockade by cGMP might be on its basis. Increase of PKC activity and some conformational changes seems to occur when adenylate cyclase is inhibited and were diminished when guanylate cyclase enzyme was repressed. The influence of acetylcholinesterase effectors on erythrocyte PKC activity seems to be relevant and could be a good peripheral biochemical marker of some neurological disturbances such as on Alzheimer's disease.

INTRODUCTION

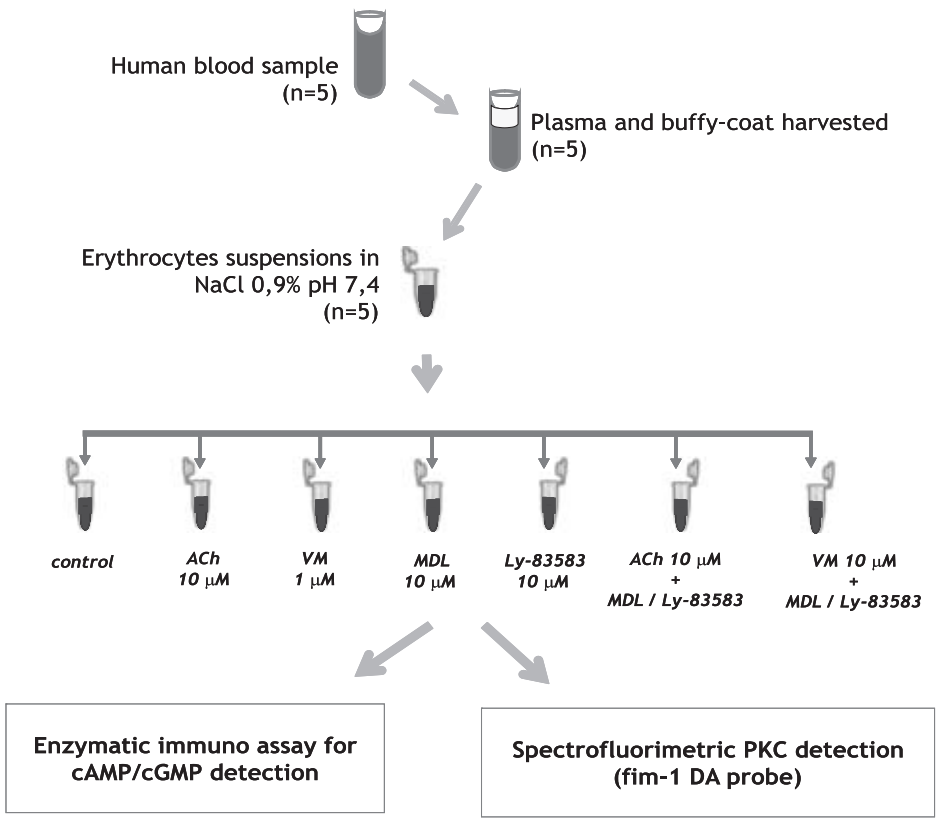
The non-neuronal cholinergic system, modulated by acetylcholine, is widely distributed in biological systems, contributing to organ and blood homeostasis[1]. A large number of pro-inflammatory and vascular effects are still under investigation.

In human erythrocytes, acetylcholine (ACh) has been proved to modulate several crucial phenomena as of hemorheological parameters and nitric oxide mobilization, with involvement of membrane-bound acetylcholinesterase (AChE)[2,3,4]. However, little has been done to correlate the influence of this dual system on erythrocyte signalling transduction pathways and the involvement of second messengers and protein kinase C (PKC). In red blood cells, PKC is known to mediate responses to calcium elevation as well as programmed cell death following glucose depletion[5,6]. In certain diseases, PKC was additionally considered as a potential marker; enhanced activity and shifts in its conformation were found in patients with chronic myelogenous leukaemia and in Alzheimer's disease, respectively[7,8].

The aim of this study was to evaluate the potential influence of acetylcholinesterase effectors in signalling pathways mediated by PKC and second messengers (cAMP / cGMP) under physiological concentrations, in human red blood cells.

METHODS

From blood samples of 5 healthy donors, erythrocyte suspensions were prepared and incubated with acetylcholinesterase substrate (acetylcholine) and inhibitor (velnacrine), and with adenylate cyclase / guanylate cyclase inhibitors (MDL and Ly-83583, respectively). The levels of cAMP/cGMP and PKC activity[9] were determined afterwards, by using an enzyme immuno assay and spectrofluorimetry, respectively.



RESULTS AND DISCUSSION

The presence of acetylcholine in ES increases cGMP and decreases cAMP, at variance when velnacrine is present enhances both second messengers. Inhibited guanylate cyclase led to lower cGMP but higher cAMP in presence of either ACh or velnacrine. By turn, inhibited adenylate cyclase let to lower cAMP in each case of effectors` presence and let to decreased cGMP with velnacrine.

Either acetylcholine or velnacrine when present in ES enhance PKC activity. A cross-talk mechanism could be proposed to explain the high levels of cAMP upon acetylcholine stimulation, inasmuch as it increases cGMP which in turn inhibits phosphodiesterase III enzyme.

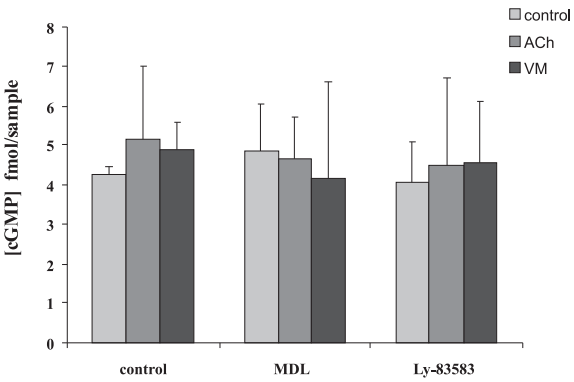


Fig. 1 – Changes on the in vitro concentration of cGMP, after erythrocytes suspensions' incubation with ACh 10 μM, VM 10 μM, in the presence or absence of MDL or Ly-83583 10 μM.

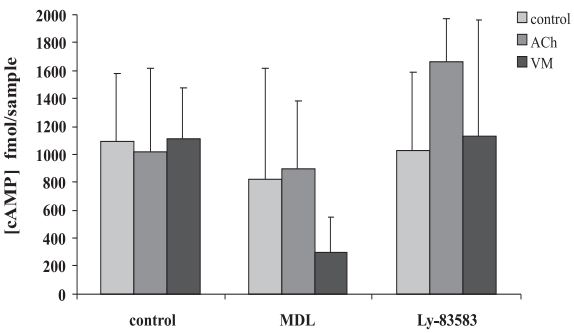


Fig. 2 – Changes on in vitro concentration of cAMP, after erythrocytes suspensions' incubation with ACh 10 μM, VM 10 μM, in the absence or presence of MDL or Ly-83583 10 μM.

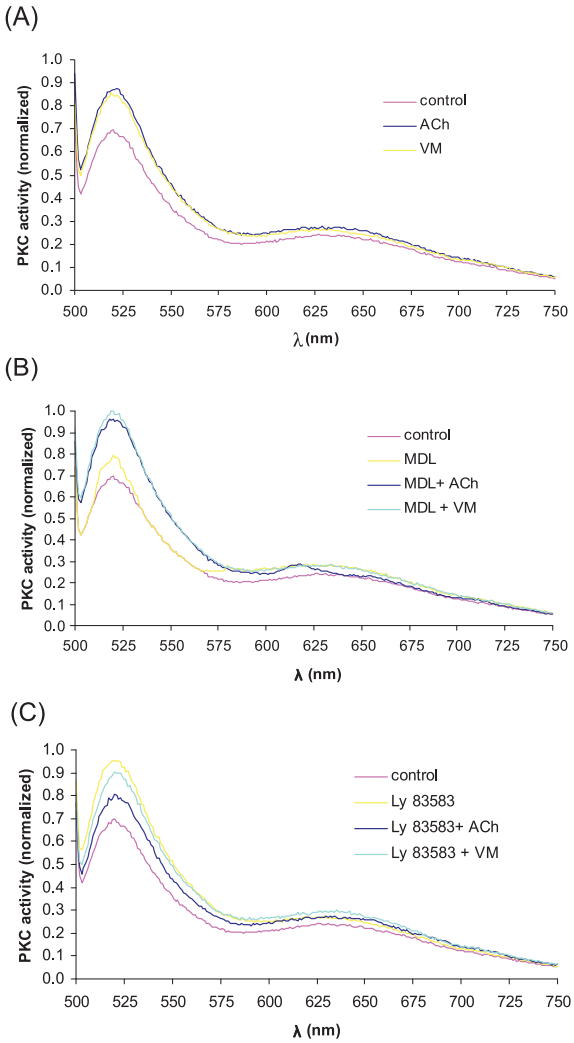
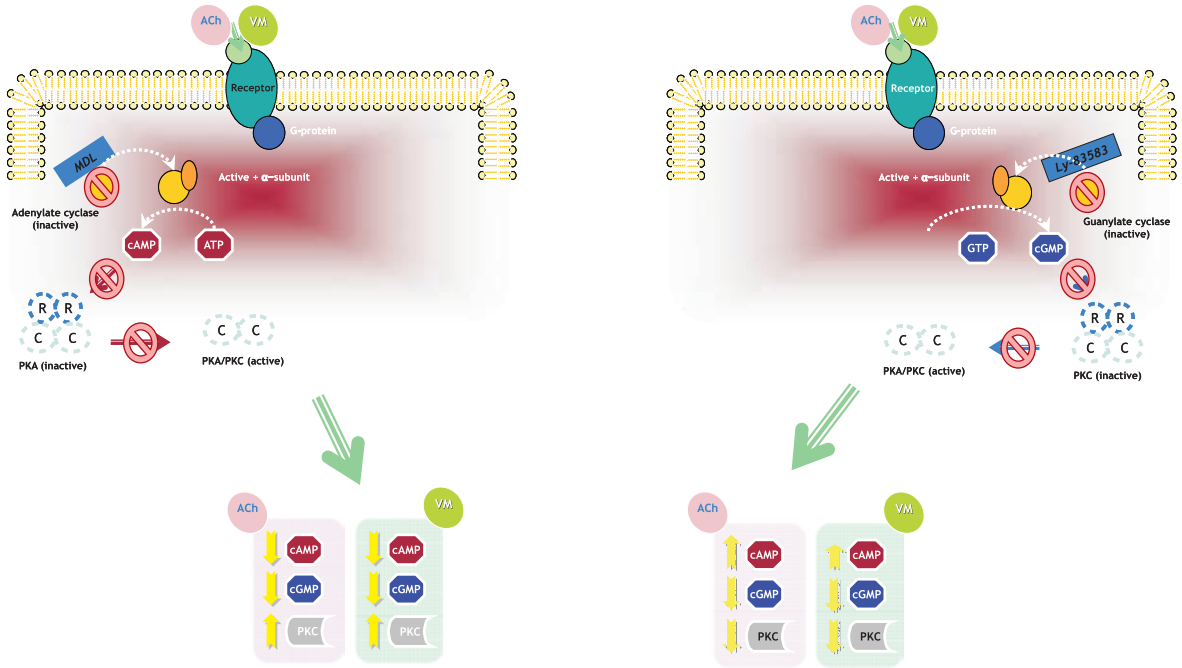


Fig. 3 – PKC activity determined by spectrofluorimetry using fim-1 diacetate probe. After 30 min at 37 ºC of erythrocytes incubation with fim-1 DA, the erythrocytes suspensions were incubated with (A) ACh 10 μM, VM 10 μM, in the absence or presence of MDL (B) or Ly-83583 10 μM (C).

CONCLUSIONS

1. Acetylcholinesterase effectors induce changes on the cAMP concentrations when in presence of either adenylate cyclase or guanylate cyclase inhibitors.
2. It seems that PKC activity is influenced by acetylcholinesterase effectors and not by adenylate cyclase and guanylate cyclase activity.



3. Both ACh and VM increase PKC activity when AC inhibitor is present and decrease with GC inhibitor.
4. This study assigns to the acetylcholine-acetylcholinesterase binomium a central role in regulating signaling pathways that involve PKC and cross-talk mechanisms among second messengers.

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SIGNALING TRANSDUCTION PATHWAYS IMPLICATED IN NO PRODUCTION IN ENDOTHELIAL CELLS TREATED WITH A SELECTIVE B_1-ADRENERGIC RECEPTOR ANTAGONIST

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INTRODUCTION

Some of vascular endothelial dysfunction are caused by an impairment in nitric oxide biodisponibility, involving resistance arteries and leading to increase the systemic blood pressure and thus to rise hypertension. Hence antihypertensive agents that reverse endothelial dysfunction and lowers blood pressure might improve the prognosis of patients with hypertension. Many studies investigated the underlying mechanism of Nebivolol® NEB-induced vasodilation and reported that is a highly selective β1 adrenoceptor blocker with additional vasodilating properties, but the mechanism that mediates these actions remain controversial. This review presents an overview of the NEB signaling pathway. Many hypothesizes in the literature suggest an endothelium-dependent liberation of NO induced by NEB in static conditions, and this NO production is due to stimulation of β_3-adrenoreceptors and oestrogen receptors and was dependent from e-NOS activation so implicating PI3-K and Akt signaling pathway. Other findings reported an endothelium independent mechanism so independent from Ca^{+2}/Calmodulin, e-NOS and β-adrenoreceptor pathway. Our studies concern the endothelium-dependent liberation of NO induced by NEB. We found that NEB induces up regulation of NO production in dose dependent manner. This increase in NO production is accompanied with a translocation of e-NOS from cellular membrane to peri nuclear region of the cell, these results suggest that hypertensive properties of the NEB are dependent from an e-NOS mechanism thus from an endothelium dependent mecanism.

ENDOTHELIUM DEPENDENT MECHANISMS

β adrenoreceptor dependent mechanism

In contrast with most of the β adrenoreceptor blockers, NEB* has vasodilatory properties in dose dependent manner in canine coronary and carotid arteries that are dependent on the presence of the endothelium and are associated with activa-

tion of e-NOS[1]. this endothelium-dependent relaxation is prevented by nitro-L-arginine, an inhibitor of e-NOS. There is ongoing discussion regarding the receptor responsible for the NEB-induced activation of e-NOS. Many finding showed that NEB activates β3 adrenoreceptor and 5HT receptor and that NEB induced e-NOS activation is due to a translocation as well as phosphorylation of the enzyme[2,3,4]. De Groot and al[5] and Kakoki and al[6] reported that the 5HT blocker (NAN 190) was able to inhibit vasorelaxation by NEB. Our in vitro results are in agreement with these findings, we found that treatment of endothelial cell monolayer with NEB up regulate NO production in dose dependent manner and causes a translocation of e-NOS from cellular membrane to peri nuclear region. Other studies have contraries these results, Chlopicki and al[7], reported that NEB-induced coronary vasodilation is not mediated either by β adrenoreceptor or by 5HT receptors, they reported that NEB conserve its vasodilatory properties by increasing in coronary flow after inhibition of $\beta_{1,2}$ and β_3 adrenoreceptor with 10^{-5} M of nadolol and 10^{-6} M of 748337 respectively. Gosgnac and al[3] have analyzed signaling pathway involved in HUVECs response to NEB, they found that NEB possesses β3 agonist properties which at the origin of its vasodilatory effects and involved phospholipase A2 and the adenylate cyclase. They evaluate the effect of antagonists of β adrenoreceptor subtypes on the NEB-induced increase in cyclic AMP concentration and found that NEB is not β1 or β2 agonist, because neither propanolol (β1 and β2 antagonist) nor butoxamine (β2 selective antagonist) blocked the induced rise in cyclic AMP concentration, treatment of ECs with bupranolol (β1, β2, β3 antagonist adrenoreceptor) or cyanopindolol (β3 antagonist adrenoreceptor) abolished the NEB-induced increase in cyclic AMP concentration. In addition it has been demonstrated that the β3 adrenoreceptor is expressed in endothelial cells from canine coronary arteries and mediate peripheral vasodilation[3]. These results are not in according with Evangelista and al[8] finding, reported that nadolol (β1 and β2 antagonist) inhibited the NEB activity on NO release, e-NOS activity and intracellular Ca^{+2} release, but SR59230 (selective β3 antagonist) significantly reduced it at a greater extent. Nevertheless, NEB in the presence of the selective β3 antagonist still induced a NO release of about 30-40% indicating the competitive nature of the antagonism and the multiple target activation induced by the drug. They suggest that this cell response is dependent from NEB concentration. so NEB at 10μM (concentration used at their study) is able to bind both β2 and β3 adrenoreceptor. Nevertheless, how β3 agonists are able to increase this NO production is still unclear.

e-NOS dependent mechanism

Apart from a discussion about which receptors are involved in NEB-induced e-NOS activation, nothing is known about the molecular alteration (phosphorylation/translocation) NEB induced on the e-NOS molecule. Several mechanisms for e-NOS activation have been discussed. Under basal conditions, e-NOS is located at the cellular membrane close to caveolin. Upon receptor stimulation, the caveolin/e-NOS interaction is released and e-NOS may translocate from the cell membrane to the cytosol[9]. In addition to translocation, e-NOS activity can be altered by phosphorylation. It has been observed that simultaneous phosphorylation of e-NOS at serine ^{1177} and threonine ^{495} may be associated with a decline in NO liberation because e-NOS is activated by dephosphorylation at threonine ^{495} and phosphorylation at serine ^{1177} [1].

Ca^{+2}/Calmodulin involvement

Gosgnac and al[3] reported that NEB induced NO over expression is not due to a Ca^{+2}/calmodulin sensitive regulation of the e-NOS activity, because it did not induce changes in the intracel-

lular Ca^{+2} concentration. These results agree with an in vivo study demonstrating that a calmodulin inhibitor did not abolish the NEB-induced vasodilation of canine coronary arteries[10]. In contrary, Parenti and al[11] showed that the NO production induced by NEB was blunted by the endoplasmique reticulum Ca^{+2}ATPase inhibitor, thus indicating that this effect was dependent on the intracellular Ca^{+2} release. Dessy and al[2], in bovine aortic endothelial cells, confirmed that NEB induces an increase in Ca^{+2} signal and NO production. Similarly, Broeders and al[12] reported that plasma of NEB-injected mice induces the release of NO from mouse aorta and augments the free [Ca^{+2}] in endothelial cells. We presently do not have an explanation for this discrepancy which might be due to the different experimental conditions utilized.

ENDOTHELIUM INDEPENDENT MECHANISM

A number of in vitro and in vivo studies have shown that endothelium-derived NO accounts for the vasodilator response to NEB[11,13,14]. Ignarro an al[4] have studied the response of rat aortic rings to NEB and compared it to the response of human canine coronary arterial and canine pulmonary arterial rings with and without endothelium. They found that coronary and pulmonary arteries relaxed in response to NEB largely by an endothelium-dependent mechanism, whereas rat aorta relaxed in response to NEB by both endothelium dependent and endothelium independent mechanisms. They suggest that the magnitude of dependence on the endothelium for vasorelaxation may vary among mammalian species. Moreover, they found that in both endothelium dependent and endothelium independent mechanisms, the NEB-response involves NO and cyclic GMP, because the vasorelaxant effect of NEB were markedly inhibited by methylene blue and ODQ (1H-[1,2,4] oxadiazolo [4,3-α] quinoxalin-1), these later are chemical agents which block the activation of guanylate cyclase (GC) induced by NO. In the present time, the nature of involvement of NO and cyclic GMP in endothelium independent mechanism remain unknown. However, they suggest one possible mechanism, is that NEB stimulates rat aortic smooth muscle cells to generate NO by an unknown mechanism, this mechanism can not be e-NOS dependent because only endothelium dependent vasorelaxation in response to NEB was inhibited by N^G-methylarginine (an e-NOS inhibitor), these results are according with our finding, that N^G-methylarginine inhibit the NEB-NO induction in static conditions. Previous studies describing the existence of a muscle derived relaxing factor (MDRF) that possess pharmacological and chemical properties similar to these of authentic NO in bovine pulmonary artery. MDRF was generated by perfusion of endothelium-denuded bovine pulmonary artery. Moreover, they found these endothelium-denuded arterial rings relaxed in response to L-arginine and that relaxant responses were accompanied by increases in smooth muscle levels of cyclic GMP and nitrite[15].

CONCLUSION

NEB represents an unique β1 adrenergic receptor blocker with vasodilatory properties. A lot of studies ported on elucidation of signaling pathway and targets receptors which responsible of this vasodilatory action. Results from diverse works leads to conclude that NEB does not have only one signaling pathway. NEB action can be dependent or no from endothelium, so dependent or no from e-NOS activation and Ca^{+2} /calmodulin complex. But the strange is that some is the signaling pathway involved in NEB action it always induce NO up regulation. However signaling pathway involved in NEB action remain unclear and several studies still continue to seek the origin of this action.

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HEMORHEOLOGIC PARAMETERS IN ANIMAL MODELS OF HYPER AND HYPOCHOLESTEROLEMIA AND HYPERTENSION

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ABSTRACT

Animals are often used nowadays as scientific investigation models for many kinds of diseases and the experimental results obtained have served as the basis for many clinical trials. Different experimental animal models (EAM) are described in the literature and choosing an appropriate model to answer our questions is always a challenging. To contribute to a better understanding of the biophysiological properties underlying the EAM we performed a hemorheological characterization of different EAM namely, hyper/hypocholesterolemia and systemic arterial hypertension.

The hypocholesterolemia model was achieved with a daily ingestion of low fat milk enriched with phytosterol esters, and a hypercholesterolemia profile was obtained with the ingestion of low fat milk with no food ingredient addition. We demonstrated that the used of phytosterols as food ingredient reduces the plasma concentrations of cholesterol and LDL-cholesterol, not affecting the HDL-cholesterol levels. During the experiment no clinical changes and no significant differences in growth, food or milk comsumption were observed. Blood and plasma viscosity, erythrocyte deformability and membrane fluidity were determined in all animal groups. The hypercholesterolemic profile is characterized by a decrease in the blood viscosity and in the erythrocyte deformability and no changes in the plasma viscosity. In the hypocholesterolemic profile achieved with the ingestion of phytosterols esters as food ingredient no significant changes were observed in the hemorheological parameters studied.

The animal model of systemic arterial hypertension is achieved with a daily ingestion of a NO sintase (NOS) inhibitor (L-NAME) for 21 consecutive days, which results in an increase of the systolic and diastolic blood pressures. Concerning the hemorheological parameters determined we observed that NOS inhibition has no effect on the erythrocyte membrane fluidity, but decreases the erythrocyte deformability. In the blood and plasma viscosities, also determined, no variations were observed in the blood viscosity, but a higher plasma viscosity is obtained in this hypertensive model.

The hemorheological characterization of an animal model is important both for its use in further experiments and its possible information for clinical trials.

INTRODUCTION

Plant sterols have been shown to reduce cholesterol absorption by blocking the absorption of cholesterol from the gut. The accepted mechanism is considered to be competition between cholesterol and the phytosterols for the micelar solubilization 12. Thus, the phytosterols' cholesterol lowering properties can have a beneficial effect on health by reducing the risk of developing artherosclerosis and coronary heart disease 3. Abnormal hemorheological properties have been described in hypercholesterolemic patients as influent factors on blood flow already disturbed by atherosclerotic plaques. Hemorheological studies conducted with hypercholesterolemic patients have shown decreased erythrocyte deformability. Thus, in the basis of the phytosterols' use as functional food, we have conducted a 30-day feeding study in rats, using low-fat milk containing phytosterols, in order to evaluate the plasma cholesterol concentrations after the 30-day feeding period as well as erythrocyte hemorheological properties.

Hypertension is the most common cardiovascular disease and is a major public health issue in developed as well as developing countries. Although, it is common to often lead to lethal complications if left untreated. Because of its high incidence and morbidity, various classes of drugs have been advocated for the control of hypertension. Human essential hypertension is a complex, multifactorial, quantitative trait under polygenic control. In order to understand the pathogenesis and to study the treatment and prevention of a disease, it is useful to develop animal models. Various models of experimental hypertension have been primarily developed to obtain information on the etiopathogenesis of hypertension. These models are also used in the pharmacological screening of potential antihypertensive agents. In the past, hypertensive animal models have been used infrequently for testing antihypertensive potential of drugs 4. As new molecules are being synthesized in a large number, the use of animal models is increasing for testing these molecules. New animal models of hypertension are being developed as new insights in to the pathogenesis of hypertension are revealed. So the characterization of an animal model is of extremely importance for its use in future studies. In order to contribute to a better understanding of the biophysiological properties underlying an animal model we performed a hemorheological characterization of an animal model of systemic arterial hypertension.

METHODS

Hypertension model

Test animals

The animals used in this study received human care in accordance with the Directive of the European Community nº86/609/CEE that mentions the protection of animals used for economic and other scientific ends. Groups of Sprague-Dawley male rats with a average weight of 238±7g, were kept in an animal facility with a 12h light/dark cycle and housed in cages in a temperature controlled room. All animals were kept on a diet standard rat food and water ad libitum for a one-week adaptation period. The animals were divided in two different groups: a Control group and L-NAME group that received L-NAME 600mg/L in water during 21 consecutive days. During the experiment, daily observations were performed.

Blood samples

At the end of the 321 days drinking period, the animals were anesthetized intraperitoneally with 1.5g/kg body weight urethane (Sigma-Aldrich) and intramuscular 50mg/Kg body weight ketamine (Pfizer, Parke Davis) after 20 minutes. Body temperature was maintained between

35-37ºC with an auto-regulated heating platform. The left carotid artery was cannulated with polyethylene tubing for blood pressure measurements and blood samples collection.

Intracellular NO and Ca^{2+} erythrocyte concentrations, erythrocyte membrane fluidity and deformability and AChE erythrocyte activity were determined, as well as plasma and blood viscosity 0 6.

Hypo and Hypercholesterolemia model

Test animals

The animals used in this study received human care in accordance with the Directive of the European Community nº86/609/CEE that mentions the protection of animals used for economic and other scientific ends. Groups of Wistar male rats (HsdBrlHan:Wist, Harlan Iberica) with a average weight of 223.67±38g, were kept in an animal facility with a 12h light/dark cycle and housed in cages in a temperature controlled room. All animals were kept on a diet standard rat food and water ad libitum for a one-week adaptation period. After this adaptation period the rats were housed singly and started drinking, instead of water, low-fat and half-fat milk containing phytosterols in tree different concentrations: 0.2g/dL (Group 1); 0.3g/dL (Group 2) and 0.4g/dL (Group 3) for 30 consecutive days, a milk group as also used in this experience using milk without phytosterols addiction. A control group was included in this study by having 10 Wistar male rats drinking water for a 30-day period. Clinical observations, body weights and milk consumption were daily measured.

Blood samples

At the end of the 30 days milk-drinking period, the animals were anesthetized intraperitoneally with 1.5g/kg body weight urethane (Sigma-Aldrich) and intramuscular 50mg/Kg body weight ketamine (Pfizer, Parke Davis) after 20 minutes. Body temperature was maintained between 35-37ºC with an auto-regulated heating platform. The left carotid artery was cannulated with polyethylene tubing and blood samples were collected.

Total cholesterol, LDL-Cholesterol and HDL-Cholesterol plasma concentration were determined using enzymatic-colorimetric tests (Spinreact, SA, Spain). With the blood samples anticoagulated with sodium heparin the following parameters were determined: erythrocyte acetylcholinesterase activity, erythrocyte membrane fluidity and erythrocyte deformability 0 6. Blood samples anticoagulated with K_3EDTA were centrifuged at 1200 rpm for 1 minute, and the resulting plasma was collected for the determination of plasma viscosity with the Harkness method 7.

Whole blood viscosity (WBV) was determined in a Brookfield digital viscometer model LVTDV II cp., using native blood aliquots submitted at low (22.5 s^{-1}) and high (225 s^{-1}) shear stress forces.

RESULTS AND DISCUSSION

Hypertension model

The systolic pressure of HTA rats is significantly augmented in relation with the control group, and, as expected low NO levels are obtained.

The erythrocyte elongation index is significantly decreased in the hypertensive rats which means that NOS inhibition leads to less deformable erythrocytes.

For the blood viscosity no significant variations were obtained and neither for the erythrocyte membrane fluidity, but a higher plasma viscosity is observed in our L-NAME induced systemic hypertension animal model. The systemic hypertension animal model is characterized by less

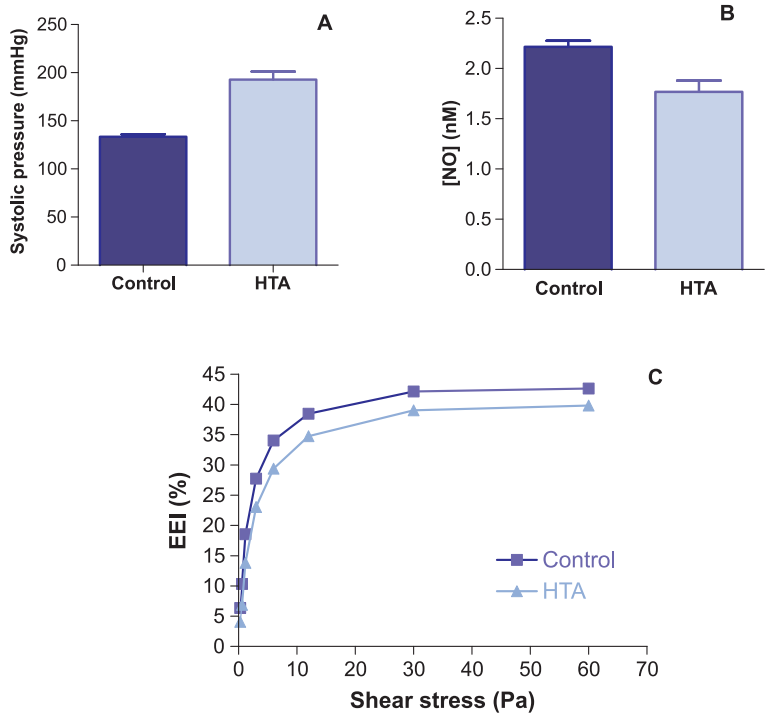

Fig. 1 – Mean and standard deviation values for the systolic blood pressure (**A**) and NO concentrations (**B**) in the control and HTA groups. High levels of systolic blood pressure in the HTA group correspond to low NO concentrations. Erythrocyte elongation index (**C**) of the two experimental groups shows that L-NAME induced hypertension leads to less deformable erythrocytes.

Table 1 – Mean and standard deviation of plasma, blood viscosity and erythrocyte membrane fluidity in the control and HTA group.

Parameters		Control	HTA
Plasma viscosity (mPa.s)		1.09±0.05	1.20 ±0.01
Blood viscosity 225s-1 (mPa)		5.20±0.42	5.28±0.07
Erythrocyte membrane fluidity	DPH (sd)	0.30±0.06	0.30±0.02
	HC (sd)	0.32±0.02	0.31±0.02
	TMA (sd)	0.33±0.05	0.27±0.003

deformable erythrocytes and higher blood viscosity, low NO levels and no significant changes on erythocytes membrane fluidity.

Hypo and Hypercholesterolemia model

The ingestion of low-fat milk supplied with phytosterols by Wistar rats and the analysis of the hemorheologic and biochemical parameters, show two different things. One is that the phytosterols when incorporated in milk maintain their cholesterol-lowering properties, as we can see by the decreases observed in the LDL levels after the milk 30-day feeding period (Figure 2).

The ingestion of milk without phytosterols leads to an increase in the LDL concentrations of about 54.5% ($p<0.007$), not affecting the cholesterol and HDL concentrations.

In the hemorheologic profile some significant differences appear comparing with the water group: a loose of the erythrocyte membrane flexibility, which can be explained by the decrease of the cholesterol in the erythrocyte membranes; and an increase of the erythrocyte deformability. Regarding the macrorheological parameters, blood and plasma viscosity, the results show a significant increase of the plasma viscosity in the ex-

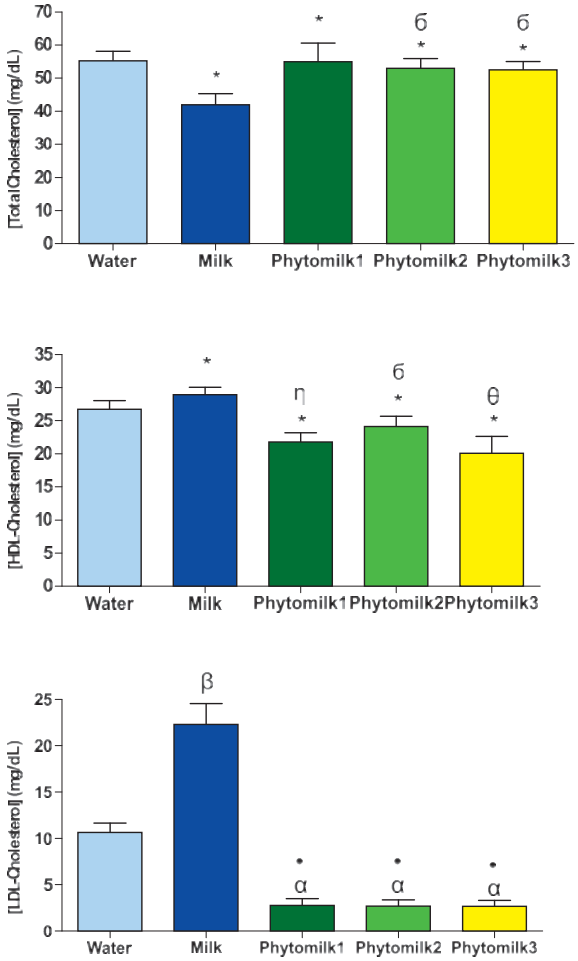

Fig. 2 – Mean values and standard deviation of total cholesterol (A), HDL (B) and LDL (C) concentrations in rat blood samples after a 30-day drinking period of low-fat milk supplied with phytosterols. In relation with the Water group: * NS; α $p < 0.001$ and β $p < 0.007$; In relation with the Milk group: ∂ $p < 0.02$; η $p < 0.01$; θ $p < 0.009$ and • $p < 0.0001$.

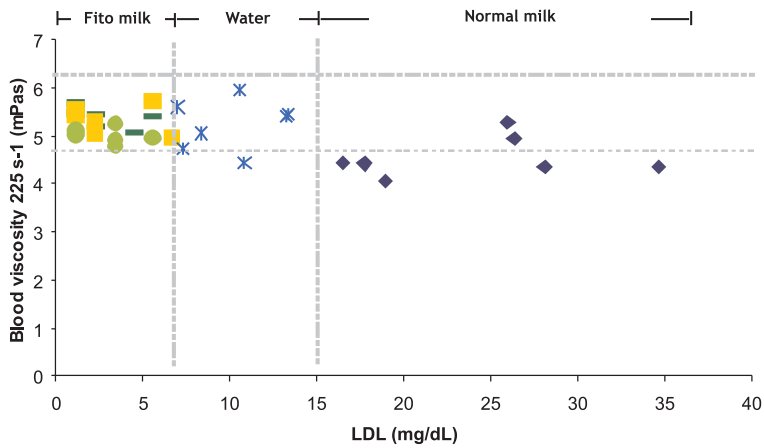

Fig. 3 – Correlation between the blood viscosity and the LDL-cholesterol plasma concentrations.

perimental groups that ingested milk with and without phytosterols incorporated and a decreased blood viscosity in the absence of phytosterols.

The results show that milk decreases the blood viscosity values but with the phytosterols ingestion the values are similar to those in the water group. With these results we may infer that milk is not well tolerated by the rat metabolism and that this is not a good hypercholesterolemic animal model.

CONCLUSIONS

In our animal model of hyper and hypocholesterolemia phytosterols when incorporated in milk maintain their cholesterol-lowering properties

Table 2 – Hemorheological parameters in Wistar rats (mean ± s.d.) determined after a 30-day feeding period with low-fat milk containing phytosterols.

	Experimental groups				
	Water	**Milk**	**Phytomilk1**	**Phytomilk2**	**Phytomilk3**
Plasma viscosity (mPa.s)*	1.09 ± 0.05	1.19 ± 0.12 NS•	1.16 ± 0.07 $p < 0.004$•	1.19 ± 0.07 $p < 0.0004$•	1.15 ± 0.06 $p < 0.003$•
Blood viscosity 225 s^{-1} (mPa)	5.20 ± 0.42	4.55 ± 0.42 $p < 0.005$•	5.36 ± 0.27 NS• $p < 0.001$°	5.54 ± 1.10 NS• $p < 0.02$°	4.97 ± 0.20 NS• $p < 0.04$°

• statistically significant difference from the Water group; ° statistically significant difference from the Milk group.

without alterations on food and milk consumption, body weight gain or biochemical parameters. Regarding the blood and the plasma viscosity, the results show a significant increase of plasma viscosity with milk ingestion and a decrease of blood viscosity for high levels of LDL-cholesterol.

The systemic hypertension animal model is characterized by less deformable erythrocytes and higher blood viscosity, low NO levels and no significant changes on erythocytes membrane fluidity. The hemorheological characterization of an animal model is important both for its use, or not, in further experiments and its possible information for clinical trials.

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HAEMORHEOLOGICAL CHANGES DURING RECOMBINANT HUMAN ERYTHROPOIETIN THERAPY IN A RAT MODEL OF RENAL FAILURE INDUCED BY PARTIAL NEPHRECTOMY

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ABSTACT

The aim of this work was to study the effect of recombinant human erythropoietin (rhEPO) therapy on haemorheological parameters, by using a rat model of chronic renal failure (CRF) induced by partial (3/4) nephrectomy.

The study used adult male Wistar rats and was performed in three groups: a control one (n=6) and in two groups with induced chronic renal failure (n=9), being one of them submitted to rhEPO therapy (n=4). Blood samples from the control group were collected at the beginning and at the end of the experimental procedure and from CRF rats at 3, 5, 8, 12 and 15 weeks after surgical partial nephrectomy. Haemorheology and renal function were evaluated.

Three weeks after the 3/4 nephrectomy, a statistically significant increase in serum urea and creatinine concentrations were found. This increase in renal function markers remained high along the 12 weeks of experimental procedure.

Comparing with controls, rhEPO treated rat have showed a statistically significant progressive increase in haemoglobin (Hb), haematocrit (Ht), red blood cells (RBC) count, mean cell volume

(MCV), mean cell Hb (MCH) and red cell distribution width (RDW), showing at 12 weeks an inverse change, though still presenting significant higher values; a decrease in platelet counts, during the first 9 weeks of rhEPO therapy. When comparing haemorheological data from non-treated CRF and controls, we found only a trend to increased MCV and MCH values and a decrease in reticulocyte count. Comparing the two groups of CRF rats, we found that rhEPO treated rats presented significantly higher values in RBC, Hb, Ht and RDW. In both groups of CRF rats, at five weeks, there was a decrease in their values, showing at the end a significantly lower value when compared to controls. No consistent alterations were found in white blood cells in CRF rats, with or without rhEPO therapy.

Partial nephrectomy seems to be a suitable methodology to induce CRF in rats and to study erythropoiesis biology. The rhEPO therapy is associated with an increased erythropoietic stimulation and a decrease in platelet count.

Key-Words: rhEPO, Erythropoietin, renal failure, rat model, Haemorheology.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone produced by kidneys, which regulates the proliferation, differentiation and maturation of erythroid cells. Chronic renal failure (CRF) patients develop anaemia due, mainly, to the low production of EPO by kidneys. To treat this anaemia, recombinant human EPO (rhEPO) therapy is currently used in these patients[1-3].

The aim of this work was to study the effect of rhEPO therapy on haemorheological parameters, by using a rat model of CRF induced by partial (3/4) nephrectomy.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River Lab. Inc.), 380-400g, were maintained in an air conditioned room, subjected to 12-h dark/light cycles and given standard laboratory rat chow (IPM-R20, Letica) and free access to tap water.

Study design

The study was performed in three groups: a control one (n=6) and in two groups with induced CRF (n=9), being one of them submitted to rhEPO therapy (n=4).

CRF was induced by a two-stage ($^3/_4$) nephrectomy: first, about half of the left kidney was removed by left flank incision and, one week later, the right kidney was removed through a right lateral flank incision[3-8].

After a three weeks stabilization period, four animals start rhEPO therapy (Recormon, Roche Pharmaceuticals, Auckland, New Zealand) in a dose of 50 IU/Kg/week, during 12 weeks.

Blood samples and Assays

Blood samples from the control group were collected at the beginning and at the end of the experimental procedure, and from CRF rats at 3, 5, 8, 12 and 15 weeks after surgical partial nephrectomy.

In all animals (controls and CRF) renal function was evaluated by determination of serum levels of urea and creatinine. Red blood cells (RBC) count, haema-

tocrit (Ht), haemoglobin concentration (Hb), haematimetric indices [mean cell volume (MCV) and mean cell Hb (MCH)], red cell distribution width (RDW), white blood cells (WBC) and reticulocyte counts were also evaluated by using a blood cell counter.

Data Analysis

For statistical analysis, we used the Statistical Package for Social Sciences, version 14.0. Results are presented as means ± SEM. Comparisons between groups at different times were performed using one-way ANOVA and Fisher's tests. Significance was accepted at p less than 0.05.

RESULTS

Three weeks after the 3/4 nephrectomy, a statistically significant increase in serum urea (71.00 ± 2.66 vs 41.00 ± 0.68 mg/dL, p<0.05) and creatinine (0.828 ± 0.036 vs 0.412 ± 0.019 mg/dL, p<0.05) concentrations were found. This increase in renal function markers remained high along the 12 weeks of experimental procedure (Fig. 1).

Comparing with controls, rhEPO treated rats showed a statistically significant progressive increase in Hb, Ht, RBC count, MCV, MCH and RDW, showing at 12 weeks an inverse change, though still presenting significantly higher values; a decrease in platelet count, during the first 9 weeks of rhEPO therapy. When comparing haemorheological data from non-treated CRF and controls, we found only a trend to increased MCV and MCH values and a decrease in percentage of reticulocyte count. Comparing the two groups of CRF rats, we found that rhEPO treated rats presented significantly higher values in RBC count, Hb, Ht and RDW. In both groups of CRF rats, at five weeks, there was a decrease in their values, showing at the end a significantly lower value when compared to controls. No consistent alterations were found in white blood cells in CRF rats, with or without rhEPO therapy.

CONCLUSION

Partial nephrectomy seems to be a suitable methodology to induce CRF in rats and to study erythropoiesis biology. The rhEPO therapy is associated with an increased erythropoietic stimulation (increase Hb, Ht, RBC count and RDW) and a decrease in platelet count. This method might be useful used to study the cellular and molecular underlying EPO resistance.

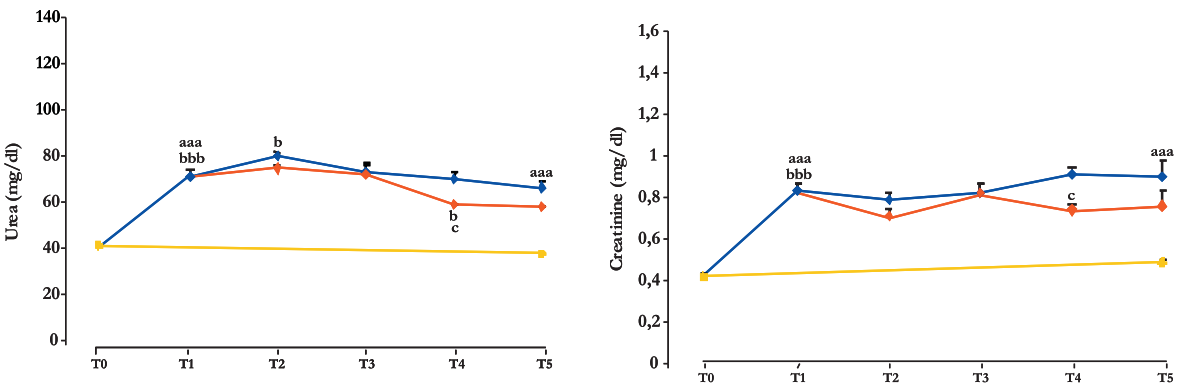

Fig. 1 – Renal function markers (urea and creatinine serum levels) along the 12 weeks of experimental procedure.
Controls; Non-treated CRF rats; rhEPO treated
a: p < 0.05, **aa:** p<0.01, **aaa:** p<0.001, non-treated CRF rats vs controls; **b:** p < 0.05, **bb:** p<0.01, **bbb:** p<0.001, T1 vs T0; T2 vs T1, etc.; **c:** p < 0.05, **cc:** p<0.01, **ccc:** p<0.001, non-treated CRF rats vs rhEPO treated rats.

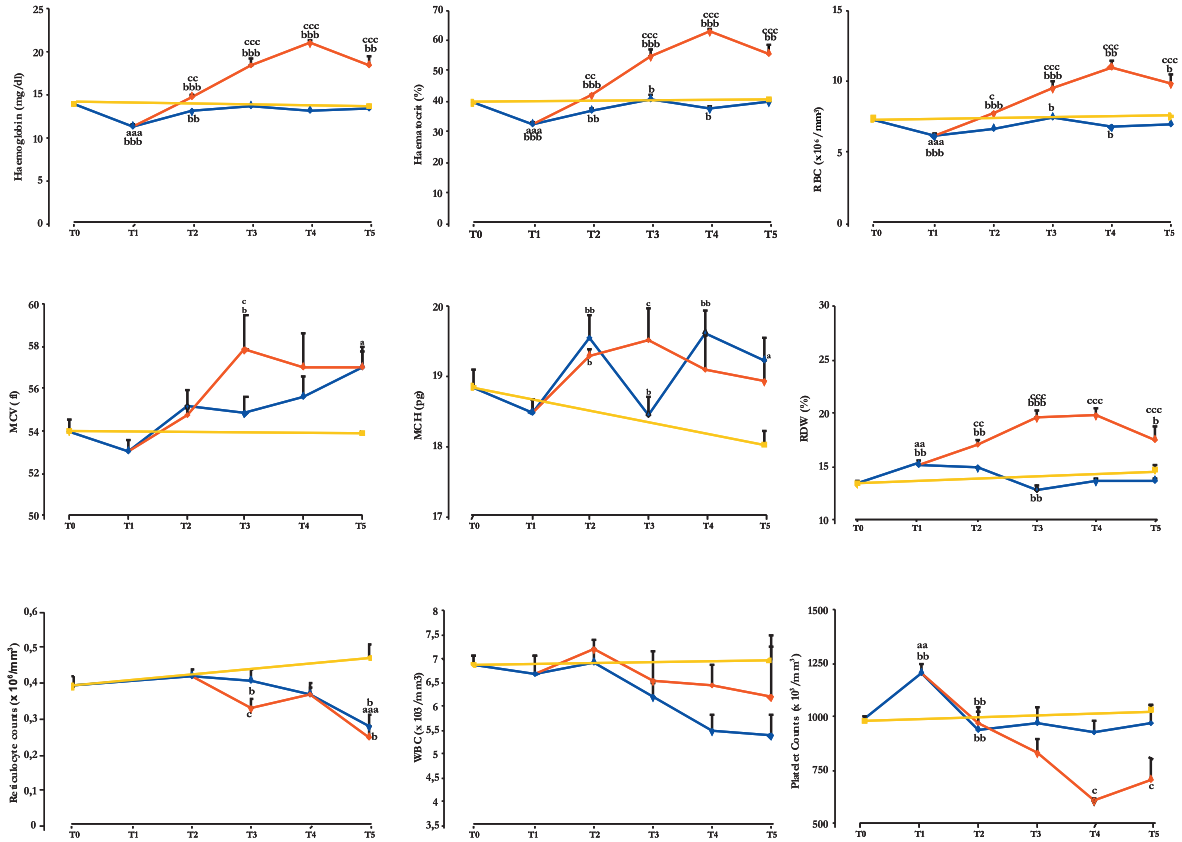

Fig. 2 – Haemorheological changes during recombinant human erythropoietin therapy along the 12 weeks of experimental procedure.

Controls; Non-treated CRF rats; rhEPO treated

a: p < 0.05, **aa:** p<0.01, **aaa:** p<0.001, non-treated CRF rats vs controls; **b:** p < 0.05, **bb:** p<0.01, **bbb:** p<0.001, T1 vs T0; T2 vs T1, etc.; **c:** p < 0.05, **cc:** p<0.01, **ccc:** p<0.001, non-treated CRF rats vs rhEPO treated rats.

ACKNOWLEDGEMENTS

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ADVANCES IN COMPUTATIONAL HEMORHEOLOGY

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ABSTRACT

Hemorheological experiments show that blood can exhibit non-Newtonian characteristics like shear-thinning, viscoelasticity and thixotropy. The rheological behaviour of blood is determined by numerous physiological factors like plasma viscosity, hematocrit or level of red blood cell aggregation and deformability, and the ability to model it is of major importance in clinical applications, where local hemodynamics plays a determinant role. A detailed discussion of different models and methods can be found e.g. in [9].

In some regions of the vascular system, as in large vessels, blood viscosity can be regarded as a constant and blood flow is well described by the Navier-Stokes equations. In other locations, the non Newtonian effects are not negligible and more complex models must be used.

The mathematical and numerical analysis of these problems is a difficult task, both from the theoretical and the computational point of view. In this paper we briefly present some relevant rheological models for blood and show numerical simulations, selected from [2,3,8,9].

Keywords: Computational rheology, hemodynamics, non-Newtonian fluid, shear-thinning fluid

INTRODUCTION

Computational hemodynamics and hemorheology is gaining more attention since cardiovascular diseases are one of the major causes of death in the world (see [6]). Local hemodynamic factors like flow separation, recirculation or low and oscillatory shear stress are recognized to play an important role in the appearance, localization and development of several cardiovascular diseases. The major role of computational fluid dynamics (CFD) is the quantification of patient specific hemodynamic parameters and also the design of enhanced prosthetic devices. With the current rate of development in the new generation of computer processors it may be possible in a near future to collect patient specific data in a non invasive way and provide in almost real time, undisturbed flow pattern simulations. In order to achieve this goal and to be able to produce realistic simulations, mathematicians, engineers, biologists and medical doctors must work together in the development of models that accurately describe the mechanical behavior of blood and blood vessels in the different districts of the vascular system. We will refer to some of these models but, for a very thorough review on this subject, we refer to Robertson, Sequeira and Kameneva[7].

RELEVANT PHYSIOLOGICAL PROPERTIES OF BLOOD

The morphology of blood determines its physical properties and consequently influences the choice of mathematical models suitable for the simulation of blood flow. Blood is a multi-component mixture with complex rheological properties, consisting of a suspension of cellular elements (erythrocytes, leukocytes and thrombocytes) in plasma, that also contains, proteins, lipids, electrolytes and other matter.

We focus attention in the erythrocytes (red blood cells), that we assume responsible for the main mechanical properties of blood. This seems a reasonable assumption since they are the most numerous of the formed elements (98%), are present in very high concentrations (aprox. $5x10^6$ RBC/mm^3) and their blood volume fraction is about 40 to 45% (hematocrit).

Red blood cells are highly flexible biconcave discs (some 8.5 μm in diameter) with a thin membrane (2.5 μm maximum thickness), filled with a saturated solution of hemoglobin (apart from cellular components). They are capable of large distortion, without changing surface area. At sufficiently low shear rates RBC's tend to aggregate in large chains called rouleaux (see Fig. 1) offering an increased resistance to motion and thus presenting a high apparent viscosity. As the shear rate increases, rouleaux break to smaller and

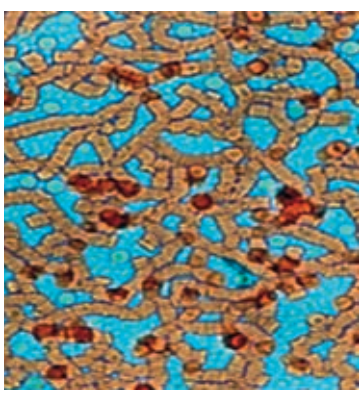

Fig. 1 – Profile view of erythrocytes forming roulleaux (courtesy of Prof. M.V. Kameneva, Univ. Pittsburgh, USA).

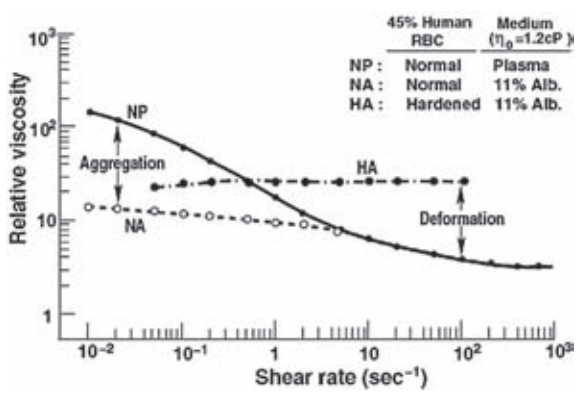

Fig. 2 – Viscosity of human blood as a function of shear rate, at Hct=45% and temperature of 36.85ºC. From Chien et. al.[4].

smaller chains until cells become individualized and finally deform and align with the main flow. For this reason the apparent viscosity of blood decreases with shear rate (shear-thinning or pseudo-plastic behaviour). In Fig. 2 it is visible the influence of the deformation and aggregation of red blood cells in blood viscosity.

Normally blood viscosity is modelled to depend only on shear rate but it is in fact very sensitive to other factors like hematocrit, temperature, gender, some pathological conditions and exercise level. For this reason special care must be taken in the choice of the experimental data that better fits to the particular situation under analysis. As an example, at constant shear rate, increasing the hematocrit from 40% to 45% can result in an increase of as much as 21% in apparent viscosity.

Some authors claim that blood has also a yield stress behavior, meaning that it requires a finite shear stress to start flowing. Experimental evidence in this direction is not completely satisfactory, in fact, due to technical difficulties in conducting experiments with blood at low shear rates, the values found for the yield stress have such a variability that definitive conclusions are difficult to fix.

Blood can also exhibit viscoelastic behavior, due to the capacity of rouleaux to store and release elastic energy. Also the deformation of individual red blood cells can play some role, but it is considered to be negligible at normal values of the hematocrit. The same experimental difficulties mentioned about yield stress make it difficult to appropriately measure the viscoelastic constants.

CONSTITUTIVE MODELLING

The basic equations describing the flow of a general fluid in a certain region Ω_t correspond to the statement of the conservation of momentum and mass

$$\rho\frac{\partial \mathbf{u}}{\partial t} + \rho(\mathbf{u}\cdot\nabla)\mathbf{u} = -\nabla p + \nabla\cdot\boldsymbol{\tau} + \mathbf{f}, \qquad \text{in } \Omega_t \subset \mathbb{R}^3$$

$$\nabla\cdot\mathbf{u} = 0$$

where the unknowns (u, ρ, τ) represent the velocity, pressure and deviatoric stress of the fluid in the current configuration Ω_t. These equations, together with appropriate initial and boundary conditions, describe the evolution of velocity and pressure in any point of the fluid domain at any time $t \geq 0$. Different materials (fluids) can be described by specifying different extra stress tensors, τ. In large vessels, where shear rate is high enough in significative regions, blood can be considered a Newtonian fluid and the equations above reduce to the Navier-Stokes equations. In this relatively simple situation the viscosity μ is assumed constant and the extra stress tensor depends linearly on the symmetric part of the velocity gradient, i.e.

$$\boldsymbol{\tau} = \mu(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)$$

If we want to incorporate the pseudo-plastic behavior described in the previous section, one must at least account for shear-dependant viscosity, which can be done simply by specifying a functional dependence of viscosity on shear rate (which is in turn computed from velocity). In this case:

$$\boldsymbol{\tau} = \mu(\dot{\gamma})(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)$$

$$\dot{\gamma} = \sqrt{\frac{1}{2}(\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T) : (\nabla\boldsymbol{u} + (\nabla\boldsymbol{u})^T)}$$

There are several usual choices for $\mu(\dot{\gamma})$, all yielding similar results, since they are designed to fit the same experimental data. In the following table we show some examples of generalized Newtonian models, together with the experimental constants, provided by Cho and Kensey[5] for blood at 37ºC. These data include both human and canine blood and the generalized Newtonian models are fit to a combined data set with a relatively wide distribution of hematocrits (33-45%).

Table 1 – Comparison of various generalized Newtonian models for blood. Experimental values taken from Cho and Kensey[5]

Model	Non-Newtonian viscosity	Model constants for blood
Power Law	$\mu(\dot{\gamma}) = K\dot{\gamma}^{n-1}$	$n = 0.61, K = 0.42$
Powell-Eyring	$\frac{\mu(\dot{\gamma}) - \mu_\infty}{\mu_0 - \mu_\infty} = \frac{\sinh^{-1}(\lambda\dot{\gamma})}{\lambda\dot{\gamma}}$	$\mu_0 = 0.056Pas, \mu_\infty = 0.00345Pas$ $\lambda = 5.383s$
Cross	$\mu(\dot{\gamma}) = \mu_\infty + \frac{\mu_0 - \mu_\infty}{(1 + (\lambda\dot{\gamma})^m)}$	$\mu_0 = 0.056Pas, \mu_\infty = 0.00345Pas$ $\lambda = 1.007s, m = 1.028$
Modified Cross	$\mu(\dot{\gamma}) = \mu_\infty + \frac{\mu_0 - \mu_\infty}{(1 + (\lambda\dot{\gamma})^m)^a}$	$\mu_0 = 0.056Pas, \mu_\infty = 0.00345Pas$ $\lambda = 3.736s, m = 2.406, a = 0.254$
Carreau	$\frac{\mu(\dot{\gamma}) - \mu_\infty}{\mu_0 - \mu_\infty} - (1 + (\lambda\dot{\gamma})^2)^{(n-1)/2}$	$\mu_0 = 0.056Pas, \mu_\infty = 0.00345Pas$ $\lambda = 3.313s, n = 0.3568$
Carreau-Yasuda	$\frac{\mu(\dot{\gamma}) - \mu_\infty}{\mu_0 - \mu_\infty} = (1 + (\lambda\dot{\gamma})^a)^{(n-1)/a}$	$\mu_0 = 0.056Pas, \mu_\infty = 0.00345Pas$ $\lambda = 1.902s, n = 0.22, a = 1.25$

Generalized Newtonian models capture the shear-thinning behavior of blood, considered its most noticeable non-Newtonian characteristic, and yet are computationally comparable to Navier-stokes equations. If the viscoelastic behavior of blood is to be incorporated, more sophisticated models must be considered, where the extra stress is no longer explicitly computed from the deformation gradient, but obtained by solving an additional (constitutive) equation. One example of such models is the Oldroyd-B model, in which the velocity and pressure are computed together with a polymeric stress component $\boldsymbol{S}_1$ by solving the coupled system

$$\rho\frac{\partial \mathbf{u}}{\partial t} + \rho(\mathbf{u}\cdot\nabla)\mathbf{u} = \mu_2\Delta\boldsymbol{u} - \nabla p + \nabla\cdot\boldsymbol{S}_1 + \mathbf{f}$$

$$\nabla\cdot\mathbf{u} = 0$$

$$\boldsymbol{S}_1 + \lambda_1\left(\frac{D\boldsymbol{S}_1}{Dt} - \boldsymbol{S}_1\cdot\nabla\boldsymbol{u} - \nabla\boldsymbol{u}^T\cdot\boldsymbol{S}_1\right) = 2\mu_1\boldsymbol{D}$$

The stabilility of this coupled system is a very important issue and poses some limitations on the efficient numerical simulation of such fluids. Usually all large scale simulations of the vascular system are performed using Newtonian or generalized Newtonian models, leaving viscoelastic models for more localized hemodynamic studies, where the dimension and/or complexity of the computational domain permits their use.

NUMERICAL SIMULATIONS

The need to perform patient specific simulations poses the problem of obtaining realistic geometries, coming from medical imaging. The process of segmenting and reconstructing 3D models from real medical images, as well as the generation of computational meshes suitable to apply numerical algorithms, is very complex and a tremendous challenge to graphic computation.

Starting from a geometrical reconstruction of the area of interest, several numerical methods can be used to solve the equations in the chosen model. The discretization in time is done by some finite difference scheme, while discretization in space can be done using finite elements, finite differences, finite volumes, mesoscopic or kinetic methods, among others.

The choice of the numerical method should be based on the flow characteristics and the complexity of the domain, in order to obtain accurate and robust results. Figures 6, 7 and 8 show numerical results using these methods applied to different geometries.

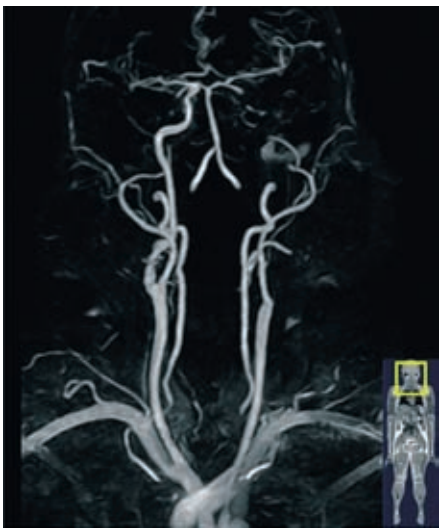

Fig. 3 – MRA of neck and brain vasculature.

Fig. 4 – Cerebral vasculature re-constructed from MRI .

Fig. 5 – Computational mesh of a segment of an artery in the circle of Willis.

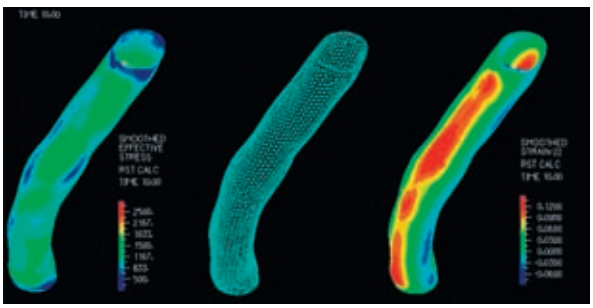

Fig. 6 – FSI Simulation in a section of the left middle cerebral artery, using FEM: wall shear stress (left), geometry and mesh (middle) and strain (right).

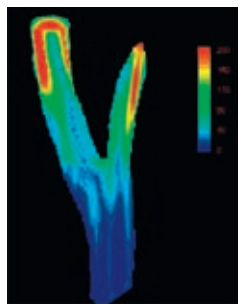

Fig. 7 – Stress com-putation on the carotid bifurcation using LBM.

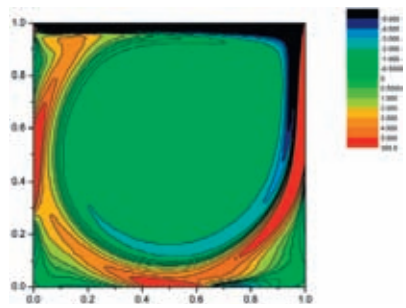

Fig. 8 – Benchmark solution (lid driven cavity) obtained with meshless methods.

The interpretation of numerical results must be done with great care, considering the limitations and accuracy of medical imaging, image segmenting and geometrical reconstruction, as well as the mathematical model used for blood flow and blood vessels, and finally of the numerical method used to perform the discretization of the problem.

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COUPLING MULTISCALE FLUID-STRUCTURE INTERACTION MODELS FOR BLOOD FLOW SIMULATIONS

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ABSTRACT

Nowadays, mathematical modelling and 3D numerical simulations play an important role in the understanding of local haemodynamics. However, the geometrical complexity of the human cardiovascular system, together with the computational costs of 3D algorithms, make it unfeasible to use them to simulate large parts of the arterial tree. Thus, truncated 3D regions must be considered, generating artificial sections where proper boundary conditions should be provided. Reduced models (1D or 0D) can be used to approximate the global circulation. These models have a lower level of accuracy, however they provide useful information that can be fed to the more complex 3D model. In this study we focus on the coupling between 3D and 1D models. We introduce the models and different strategies to couple them. We apply the coupling to a real 3D carotid geometry and a 1D network including the circle of Willis (CoW).

Keywords: mathematical models, numerical simulations, geometrical multiscale approach, fluid-structure interaction, coupling 3D and 1D models.

INTRODUCTION

Over the last years, mathematical modelling and numerical simulations of blood flow have gained a great relevance in the understanding of the human cardiovascular system, in particular the origin and development of cardiovascular diseases[1,2,3]. However, modelling the human circulatory system remains a very difficult and challenging task because of its complexity and heterogeneity. In particular, realistic numerical simulations of blood flow in arteries cannot be performed without taking into consideration the link between local and global phenomena. Moreover, blood flow is characterized by pulse waves due to the fluid-structure interaction (FSI) between blood and the vessel wall[10], which should be properly captured by the numerical models. The geometrical multiscale modelling of the cardiovascular system was introduced to deal with this complexity and diversity[4,5]. It consists of a hierarchical description, in which the different parts of the circulatory tree are approximated at different dimensional scales, 3D, 1D and 0D, corresponding to different levels of desired accuracy. At the higher level are the three-dimensional (3D) models. They describe very accurately the blood flow velocity and pressure fields, but in practice they are applied only to relatively small computational domains. On the artificial sections generated by the 3D domain truncation, one can account for the remaining parts of the cardiovascular system by means of simpler, reduced one-dimensional (1D) or lumped parameters (also called 0D) models. They are usually obtained by making simplifying assumptions and performing averaging procedures on the 3D model. One of the challenging tasks in using the geometrical multiscale approach is the setting of proper coupling conditions to exchange quantities such as the flow rate, the mean pressure or the area at the interfaces between different models. In this study we focus on the coupling between 3D and 1D models[5,6,7].

THE MODELS

As 3D blood flow model we take the Navier - Stokes equations for Newtonian and incompressible fluids, since in medium to large vessels blood can be assumed to be Newtonian[8,9]. The interaction between blood and the artery wall is taken into account by coupling the Navier-Stokes equations with the equations of 3D elasticity for St. Venant-Kirchhoff materials, which is the model chosen to simulate the structure wall[5,6]. As coupling conditions between the fluid and the structure models we impose, at the coupling interface, the continuity of the normal stresses and the continuity of the velocity[5,6]. The FSI coupling is carried out through a Newton implicit algorithm[6], sub-iterating between both models until convergence is reached.

The 1D model is derived from the 3D FSI model by making some simplifying assumptions and integrating over the cross section[4,5]. It is described by a hyperbolic system of equations and, despite having a lower level of accuracy compared to the full 3D model, it is still able to capture effectively the pulse waves characteristic of blood flow. Coupled to the 3D detailed problem, the 1D model can act as absorbing (or far field) boundary condition. Moreover, due to its low computational cost, it can be used to simulate large parts of the arterial tree[4,5].

To couple the 3D FSI and the 1D models we impose, at the coupling interface, the continuity of the normal total stresses and of the fluxes[5,6]. With this choice, the mathematical model of the coupling is stable as long as the continuity of the area is not imposed at the coupling interface[5]. Nevertheless, the numerical evidence shows that as long as an implicit coupling scheme is applied, the coupling with the continuity of the area is numerically stable for the test cases studied[6].

In order to prescribe the continuity of the normal total stresses and the fluxes at the coupling interface (see Figure 1), we propose two possible strategies. Either the 3D model provides the 1D with the normal total stress, while the 1D gives back the flow rate to the 3D model (flow rate problem) or vice-versa

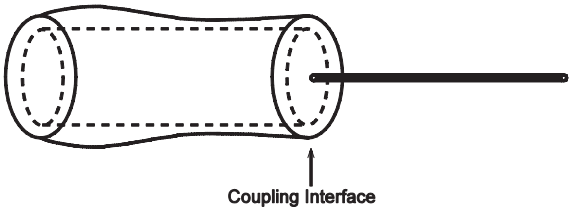

Fig. 1 – Coupling the 3D FSI and 1D models.

(mean pressure problem), see Figure 2. In both cases the 1D model provides only averaged quantities, such as the mean pressure and the flow rate, while the 3D model requires pointwise data, i.e., the normal total stress and the vectorial velocity at each point of the coupling section. In order to treat the defective boundary data coming from the 1D, special techniques must be applied. In the case of the flow rate problem, the flux condition is considered as a constraint imposed through a Lagrange multiplier[11]. For the mean pressure problem, the mean normal total stress value is imposed to the 3D through a natural boundary condition constant on the section[12].

Numerically, we propose two possible algorithms to perform the coupling[5]: one explicit, without sub-iterating at each time step and thus without reaching exactly the continuity of both the normal total stresses and the fluxes at the interface; and another implicit, based on a fixed point iteration, where the continuity of the quantities is reached within a certain tolerance.

NUMERICAL RESULTS AND DISCUSSION

We consider an anatomically 3D realistic compliant model of a human carotid bifurcation coupled with 1D models of a single tube at each downstream section. At the upstream section we impose a pressure impulse of 100mm/Hg during 0.003s. Although a single impulse is not physiologic, it is useful to highlight the properties and complexity of the problem, namely the presence of numerical spurious reflections due to the FSI phenomena.

Fig. 2 – (left) Flow rate problem. (right) Mean pressure problem.

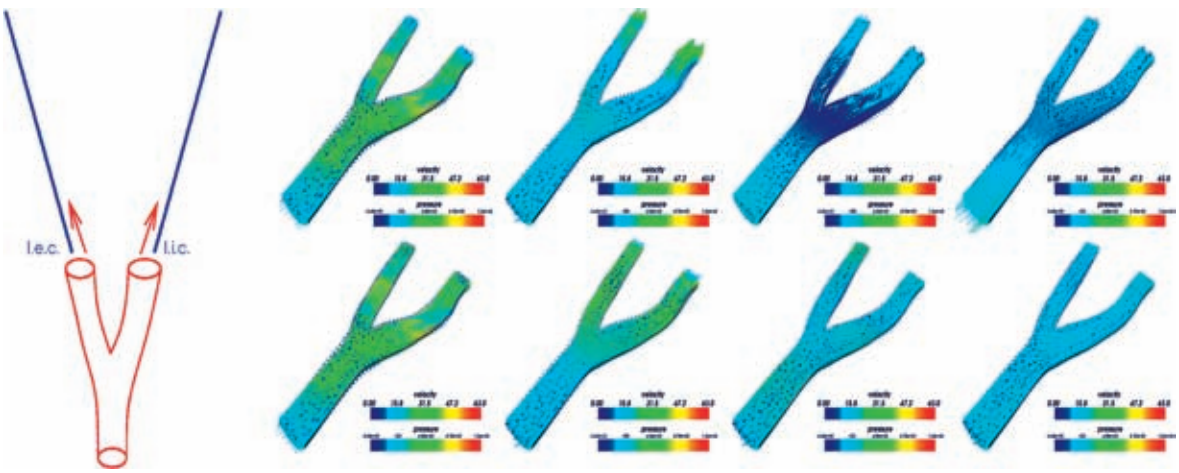

Fig. 3 – (right) Coupling the 3D model with a single 1D tube at each downstream section. (left) Comparison between the uncoupled (up) and coupled (bottom) pressure and velocity solution values at four different time steps (from left to right): 0.001s, 0.0015s, 0.002s, 0.0025s.

In Figure 3 we compare the velocity and pressure solutions on the 3D domain of the carotid bifurcation for the coupled and uncoupled problems. In the uncoupled problem we prescribe a standard free stress boundary condition at the downstream sections. We can conclude that, unlike the uncoupled solution, the coupled one does not present spurious reflections, thus the 1D model acts as an absorbing boundary condition. However completely absorbing boundary conditions are not physiologic, since bifurcations in the arterial tree produce reflections. Hence, in the next simulation we embed the 3D model into a 1D network model including the circle of Willis at the upstream of the internal carotid branch (see Figure 4, left). At the inflow of the upstream 1D model we consider the same pressure impulse as before. Although still preliminary, since a physiologic pressure impulse at the inflow is not considered, the results pictured in Figure 4 show that a bigger resistance in the internal carotid, due to the coupling with the CoW, can be perceived. This numerical test demonstrates that this coupling can be applied to general 1D models, embedding the 3D model in the global circulation. With this technique it is possible to have more realistic FSI simulations, accounting for the global haemodynamics in 3D FSI numerical simulations, which is very difficult to achieve with standard procedures or reduced models other than the 1D. Further numerical simulations on realistic cases, including physiological impulse data and other 3D realistic geometries, as well as other complex 1D networks representing large arterial trees, are ongoing.

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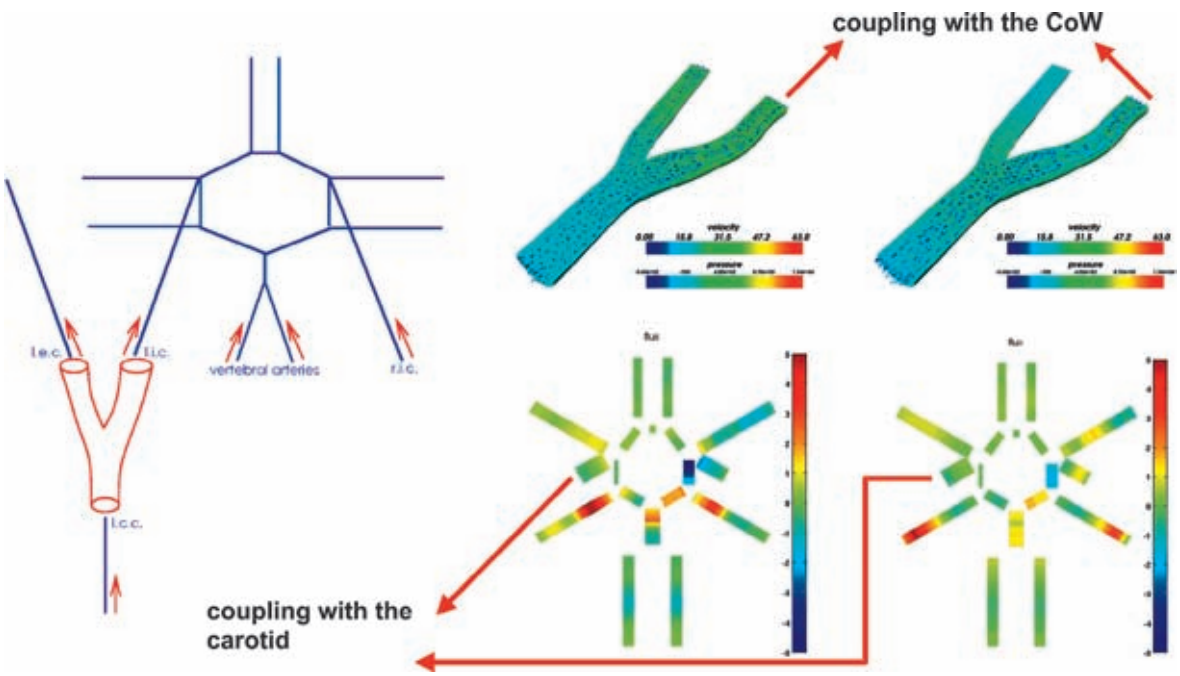

Fig. 4 – (left) Embedding the 3D model into a 1D network including the CoW. (right) Pressure and velocity solution values in the carotid bifurcation (up) and the values of flow rate in the CoW (down) at two different time steps: 0.02s (left) and 0.025s (right).

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SHEAR-THINNING DEPENDENT MECHANISMS OF LEUKOCYTE RECRUITMENT TO THE ENDOTHELIAL WALL

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ABSTRACT

We investigate the role of hemodynamical forces on the recruitment of leukocytes clustering at inflammatory regions toward the endothelial walls of post-capillary venules of Wistar rats cremaster muscles. The main objectives of the study are to quantify localized hemodynamics of blood on the margination and recruitment of leukocytes integrating experimental and simulation studies. Intravital images of leukocytes trafficking in post-capillary venules were captured and analyzed. From these images, instantaneous positions, velocities, torques and accelerations were calculated. The shear-dependent viscosity behavior was experimentally recorded and was found to follow a Carreau-Yasuda model. A novel lattice Boltzmann shear-thinning blood flow solver was used to quantify localized hemodynamics on surface of moving leukocytes as well as on the endothelial wall. We have found that the moving leukocytes strongly disturb the shear stress on the endothelial wall at large distances. Leukocytes trafficking increase the endothelial wall shear stress and create vortices and stagnation points which all act in support of the recruitment process.

Keywords: Leukocytes dynamics; intravital microscopy; computational hemorheology; leukocyte wall shear stress.

INTRODUCTION

The mechanism of margination of leukocytes from the blood main stream is not well understood and more focus on the causes and the force that initiate margination is mandatory. It is believed that rolling is mediated by adhesion molecules of the selectin family which are expressed on the leukocyte surface (L-selectin) on the endothelial wall (E-selectin) and in platelets (P-selectins). The forces applied by these mediators are not enough for marginating the leukocytes and therefore, it is necessary to study the hydrodynamic forces and their interaction with the molecular forces in detail. Leukocyte arrest, recruitment and subsequent rolling, activation, adhesion and transmigration is a multistage process which is mainly triggered by hemorheologic forces and supported by shear-induced selectins, chemoattractants and adhesion molecules on the surface of the leukocytes and the endothelial wall[1,2]. This is a multiscale process which requires more adequate sub-macroscopic models which are capable of computing localized hemodynamics such as the wall shear stress and velocity distribution. A certain density of selectins and a high tensile strength of the selectin bond are necessary for the capture of leukocytes to confirm rolling. In this study we have performed experiments on Wistar male rats and recorded leukocytes dynamics in real time using intravital microscopy. We have used these experimental data to build a shear dependent viscosity model and plug it into a lattice Boltzmann numerical solver for shear thinning flows[3]. The most important feature of the lattice Boltzmann method is its capability to compute the shear stress independent of the velocity fields, from the non-equilibrium part of the distribution functions and the possibility to capture fluid-structure interaction from momentum exchanges. It is also fully adaptive for modeling multi-component non-Newtonian flows.

MATERIALS AND METHODS

Animal Preparation

The animals used in this study received human care in accordance with the directive of the European community no. 86/609/CEE. The animals were prepared as explained in ref. [1] with the right jugular vein and the left carotid artery catheterized. The animals were prepared for intravital microscopy according to Hill[4], as illustrated by Fig.1. Intravital images of the dynamics of leukocytes were recorded and analyzed.

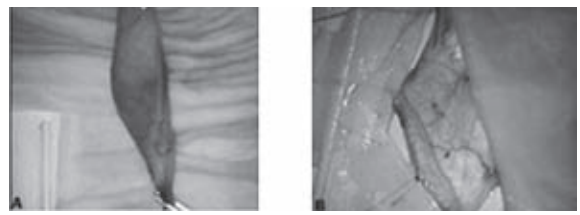

Fig. 1 – Experimental procedure of cremaster muscle preparation for intravital microscopy in a Wistar rat including isolation (left) and exposure in a support (right)

Using a Brookfield digital viscometer, the shear dependent viscosity behaviour was obtained from blood samples taken after the intravital microscopy observations.

Numerical method

We have used a recently validated lattice Boltzmann model for shear thinning blood flow[3] to simulate individuals and clusters of moving leukocytes in three dimensions. The method is mesosopic, based on the Boltzmann transport equation from physics which is suitable for solving multi-scale phenomena that commonly appear in complex fluid flows. For non-Newtonian flows, the shear rate is locally computed from the non-equilibrium parts of the distribution functions, without a need for cal-

culating velocity gradients. The drag and lift forces and the torques are easily computed from momentum exchange between the fluid and the structure[5].

The initial positions, velocities and torques were obtained from the experiment and used to initialize the solver.

Computed drags and torques were used to update the leukocyte positions. Translational and angular velocities were set on surface from the measured values.

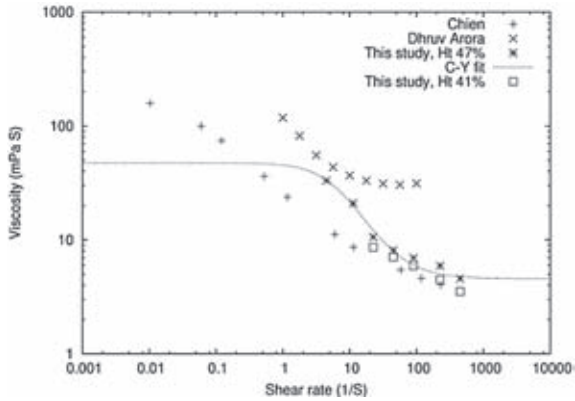

Fig. 3 – Shear rate dependent viscosity values (at different hematocrits) obtained from blood samples of Wistar rats. Shown is also the Carreau-Yasuda shear thinning fit

RESULTS

Experimental results

We have successfully tracked the dynamics of leukocytes from the in vivo experiment using the intravital microscopy. A snapshot of recorded video frames is shown in Fig. 2. From these real time frames we computed positions, velocities and acceleration of each cell. The average rolling speed was found to be 2.73 μm/s at 131 mmHg systolic pressure, 90 mmHg diastolic pressure and 394 BPM. The velocity and acceleration were computed from the measured tracks. Blood samples were taken and analyzed by a viscometer to determine the shear thinning behavior of the rats' blood (see Fig. 3).

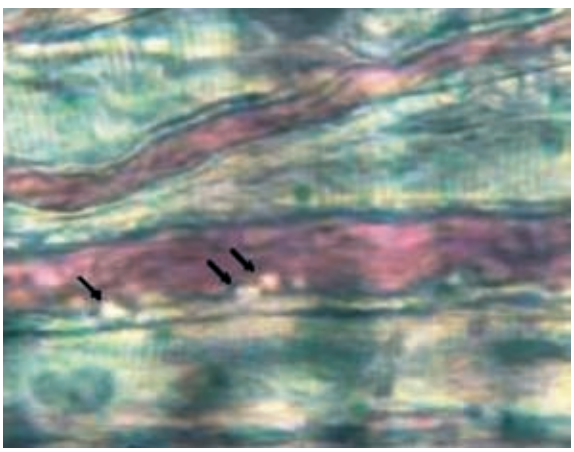

Fig. 2 – Intravital microscopy of recruited leukocytes (the white spots) in a post-capillary venule in a Wistar rat cremaster muscle. Rolling leukocytes are indicated with arrows

Numerical results

We have conducted a number of simulations for individual leukocytes, represented as irregular spheres, and for a cluster of moving leukocytes. From these simulations we have observed that the motion of leukocytes through a small enough venule results in disturbing the flow and increasing the endothelial wall shear stress at large distances. Shown in Fig. 4 is the influence of a not yet marginated leukocyte (moving through the centerline of the venule) on the endothelial wall shear stress. We suggest that the

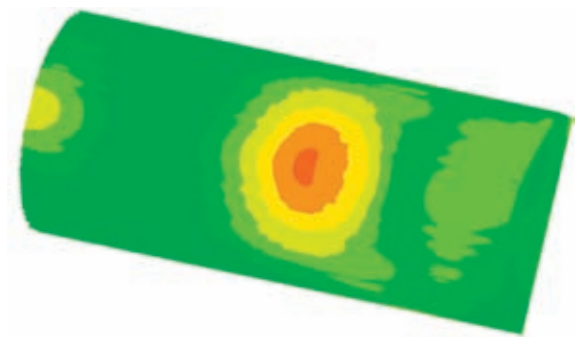

Fig. 4 – Influence of a free flowing leukocyte on the endothelial wall shear stress

endothelial wall shear stress will be high enough to activate the endothelial cell monolayer and the mediators of the selectin family, initiating the capture of the leukocytes from the blood mainstream.

We have also observed four stagnation points on the endothelial wall: two upstream and two downstream with respect to the recruited leukocyte. These stagnant regions may help in the capture of leukocytes and decelerates their motion (see Fig.5). For a cluster of recruited leukocytes, the traps and the vortexes form a helical stream which also supports leukocytes margination toward and their rolling on the endothelial wall, as it is shown in Fig. 6.

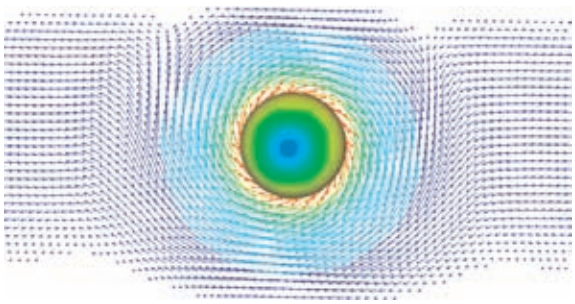

Fig. 5 – Leukocytes are trapped by four stagnant regions close to the endothelial wall (with the flow motion directed to the right)

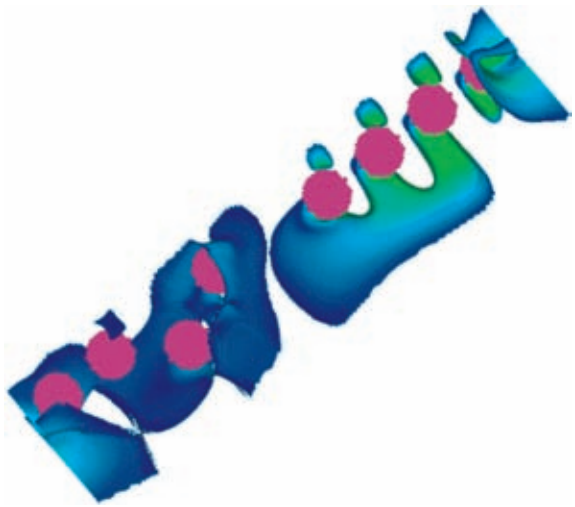

Fig. 6 – Velocity isosurface around recruited leukocytes. The white jackets correspond to stagnant regions.

DISCUSSION

During inflammation, the recruitment of leukocytes from the blood stream and their subsequent adhesion to the endothelial wall are essential stages to the immune response system. The interaction between the recruited leukocytes and the endothelial cells is a key parameter in some pathogenesis of vascular diseases. It is important to evaluate leukocytes dynamics and quantify their localized velocities on surface shear stress and to capture the influence of the resulting hemodynamics on the endothelial wall at the inflammation regions.

Intravital microscopy is an adequate routine tool used for tracking the leukocyte dynamics but is restricted to two dimensions. In this study we were able to obtain more information by conducting three dimensional time dependent simulations for flow of leukocytes through a venule. The data obtained from the experiments were used to set the initial and boundary conditions and to adopt a shear thinning viscosity model. It deserves noting that most of the studies reported in the literature (in vivo, in vitro or numerical) consider Newtonian flow dynamics of a 2D system. In this study we have demonstrated the influence of shear thinning flow on the recruitment and rolling processes of leukocytes. The role of hemorheologic forces of a cluster of leukocytes was also demonstrated. Moreover we have observed that the shear stress on the surface of a recruited leukocyte shows two regions of minimum and one region of maximum shear stress. This may play a dominant role in directing the leukocyte toward the endothelial walls. However, it is essential to consider leukocytes as deformable cells and to include the effects of erythrocytes as individuals in the recruitment process. Ongoing research includes studies on adhesion and transmigration of rolling leukocytes.

ACKNOWLEDGEMENTS

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