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Cardiovascular Biology: Endothelial Cell in Health and Hypertension

Volume I

Edited by
J.-F. Stoltz

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Welcome address – Introduction to the symposium

Ladies and gentlemen, dear colleagues and friends

It is a great pleasure for me to welcome you to this first symposium on Vascular Endothelium, NO and Hypertension, on behalf of myself, my University and the European Society on Cell and Tissue Engineering and Therapy.

This symposium is a continuation of the four previous symposia organized by our scientific society on "Mechanobiology of Cartilage and Chondrocyte" with a central thema on a current field of research.

Indeed, vascular endothelial plays a significant role in regulating blood flow, and endothelial cells (EC) have highly active metabolic functions. They generate vasoactive mediators like prostacyclin, nitric oxide (NO) and endothelin (ET-1). Endothelial cells also synthesize various proteins like von Willebrand's factor, growth factors, tissue plasminogen activator, ... They have also enzymes that inactivate bradykinin and convert angiotensin I into angiotension II (a vasopressor agent).

In other respects it is now well known that mechanical forces and stretch can modulate EC functions by activating mechano-sensors, signalling pathways, genes and protein expressions. This mechanical approach leads to a better knowledge of vascular remodelling observed in many pathologies.

These different aspects will all be studied during this first symposium.

I would like to take this opportunity to sincerely thank all the participants for their enthusiastic response to my invitation and for their outstanding contributions. I could not finish without expressing my sincere thanks to my University for its patronage which places such emphasis on the interest in these new themes of research. I would also like to thank NEGMA-LERADS Laboratories for their efficient partnership and especially Doctor Martine Burger for her important investment in the organization of this symposium.

Enjoy your stay in Prague.

J.F. Stoltz

Part I

Physiology of Endothelial Cell

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Introduction to endothelial cell biology

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Abstract. Vascular endothelial cells form a monocellular layer on blood vessel walls with an estimated mass of 1.5 kg. One of the roles of endothelial cells is to control the hemodynamics through various metabolic activities affecting homeostasis, vascular tonus, blood fluidity, coagulating properties and blood cell adhesion. In other respects thousands of studies have underlined the crucial role of local blood flow conditions on their properties. However, the hemodynamic forces are different according to the anatomical site and to the type of blood vessels (arteries, veins, venules, ...). In microcirculation, the endothelial cells in the venules are particularly active and constitute the physiological site of liquid exchange (permeability) and above all cellular transit. During critical ischemia, the post-capillary venules are deeply involved. In other respects the properties of endothelial cells may be impaired in many diseases as atherosclerosis, hypertension, inflammation and metabolic diseases.

1. Functional properties of endothelial cells

The endothelium is normally anti-thrombotic and anti-adhesive, to ensure "blood fluidity". During aggressions, the endothelium can reverse its function by expressing stored material or by slower involvement of genes which until then were repressed.

1.1. Anti-thrombotic action

The anti-thrombotic and "fluidifying" actions of endothelial cells are due to three types of properties:

(a) vaso-regulating properties: They are controlled by the release of vasomotor components as endothelin ET-1 with vasoconstricting properties on the one hand and prostacyclin (PGI2) and nitrite oxide (NO) on the other;
(b) anti-thrombotic properties: Endothelial cells express proteoglycans on their surface, including plasminogen, sulfate glycoaminaoglycans and negative-charges. They secrete plasminogen tissular activator (t-PA) and tissular factor inhibitor. One other fundamental property of the endothelium is its production and expression of thrombomodulin (a thrombin receptor). This protein promotes the formation of a complex which activates Protein C. Its function is a major anticoagulating function, controlling thrombin generation at the sub-endothelium and blood cell interface. The endothelium also exerts anticoagulant properties by other channels, such as the capture and degradation of thrombogenous substances (ADP, 5-HTP) and through the effect of active products on platelets;
(c) anti-adhesive properties: In an inactive or active state the endothelial cells express adhesion molecules, that can be modulated by mechanical or biochemical stimulations.

[*] Both authors contributed equally to this work.

1.2. Thrombotic and adhesive properties

During inflammation, the endothelial cell properties may be reversed. Variations in local shear stresses may also modify the secretion of vasomotor substances. In this case thrombomodulin is under-regulated and no longer appears on the endothelium surface. In addition, the cell expresses tissular factor that can bind to plasma VIIa factor. Hence thrombin production may occur all the more easily as factors I and X possess binding sites on the endothelium. Regarding fibrinolysis, PAI-1, an inhibitor of plasminogen activator of endothelial origin, is increased and t-PA is under-regulated.

Moreover, endothelial cells produce von Willbrand factor through the Weibel Palade bodies. In the case of endothelial lesions, von Willbrand factor will be expressed, and enhance the platelet adhesion with a binding to the platelet GPIb-IX-V complex. It was shown that the expression of von Willbrand factor is regulated by blood flow.

2. Blood cell adhesion and endothelial cells

Intercellular adhesion is a major step in the complex sequences that lead to acute or chronic inflammatory states, to thombosis and atherosclerosis or to vascular lesions. Cell adhesion is made possible by the so-called "adhesion molecules", which enable interactions between vascular endothelium cells and blood cells (leukocytes in particular). Indeed, the interactions of circulating leukocytes with the endothelium, rolling and adhesion during immune and inflammatory reactions are dependent of transient events. Three super-families of adhesion molecules are involved in these processes: Selectins, heterodimeric integrins and immunoglobulin super-family.

2.1. Selectins

The selectins (calcium-dependent and carbohydrate-binding proteins) are implicated in the initial binding of circulating leukocytes to vascular endothelium during the capture and the rolling step of adhesion in the course of inflammation. Selectins mainly recognise the ligands structures which contain fucosylated carbohydrates, and sialyl-Lewis in particular (sLE). These selectin/carbohydrate interactions are labile and enable leukocytes to "roll" along the vascular endothelium in the direction of the blood flow. There are three types of selectins: L (leukocyte)-, P (platelet)- and E (endothelial)-selectins. The main role of selectins is primarily in the interaction between leukocytes and endothelial cells.

(i) E-selectin is expressed on the endothelial cells after activation by cytokines. The peak level of expression is reached after 4–6 hours after stimulation. The expression is strong around vascular inflammation sites, in cardiac or renal graft rejection, in skin vessels near psoriasis-related superficial lesions, in systemic lupus erythematosus and in Sjogren's syndrome.

(ii) L-selectin is expressed by leukocytes on their surface and its expression is very sensitive to cellular stimulation.

(iii) P-selectin are released by Weibel–Palade bodies and by platelet granules α. P-selectin surface expression respond rapidly to a variety of agents like thrombin, cytokines, etc. and can be an excellent go-between for the mediation of the initial leukocyte/endothelium interactions.

2.2. Integrins

The integrins are transmembrane linked to the cytoskeleton, to which they transmit extracellular signals. Each integrin is a heterodimer formed by two α and β chains that are not covalently linked. So far, eight β chains and 12–15 α chains have been identified and classified. A large number of integrins belong to the $\beta 1$ subset or VLA. The $\beta 2$ chain is represented by leukocyte receptors LA-1 which is exclusively expressed on leukocytes. The $\beta 3$ subset, expressed in endothelial cells, is a platelet receptor.

Integrins permit leukocyte adhesion to endothelial cells and are involved in inflammation, cell growth, cellular differentiation, etc. Integrin LA-1 is the most implicated in leukocyte/endothelium interactions (ELAM-1: leukocyte function associated molecule-1, CD11a/CD18).

Integrins are also concerned with the endothelial cell adhesion to the extracellular matrix.

2.3. Immunoglobulin superfamily

Immunoglobulins constitute the largest family of cellular surface molecules. Their structure is characterised by repetitive domains similar to those found on immunoglobulins. Three molecules from that family play a predominant role in the mechanism of leukocyte adhesion to vascular walls and are involved in inflammatory phenomena. The most important molecules for endothelial cell/leukocyte interactions are ICAM-1, ICAM-2, VCAM-1, which act as ligands integrin LA-1 (CD11a/CD18). However, their distribution and function are different. ICAM-1 is highly expressed in endothelial cells activated by cytokines or by local shear stress. It plays a major role in inflammation and atherosclerosis. In contrast, ICAM-2 is mainly found in resting endothelial cells and leukocytes. Its expression is not enhanced by cellular activation. VCAM-1 is expressed on activated endothelial cell surface and is absent on resting endothelial cells. Its expression is regulated by an oxidative sensitive mechanism. It binds to integrins $\alpha 4$ $\beta 1$ (VLA-4). It is regulated by inflammation mediators like cytokines and is reduced when endothelial cells are subjected to shear stress.

The endothelium/leukocyte interaction processes involve a complex equilibrium between various adhesion molecules specific for a given cell type or stimulus. But on the occurrence of a stimulus with a vascular dysfunction, the equilibrium is broken and the expression of one or several adhesion molecules is modified. The study of endothelium/leukocyte interactions is therefore crucial to understand and treat dysfunctions that may occur at the vascular level.

3. Rheological properties of endothelial cells

With respect to the interfacial importance of endothelial cells, the specification of their mechanical properties is particularly relevant. However, if a lot of studies have investigated morphological modifications under flow, very few studies have been focused on mechanical forces on the deformation within the cell. Thus only one study conducted *in vitro* with confocal microscopy, during induced deformations has shown that endothelial cells behaved like an elastic isotropic material. However, deformation is less marked in the nucleus vicinity, indicating its greater rigidity. It was also shown that endothelial cells were motile in the direction of the flow, indifferently upstream or downstream.

4. Response to local flow: mechanobiology

The influence of local hemodynamic conditions on endothelial cells has raised increased interest over the recent years. Various studies have tried to clarify the physiology and modifications of EC during vascular diseases. Endothelial cells react to hemodynamic forces by modifying their morphology and metabolism. The morphological changes may include elongation and orientation of endothelial cells parallel to the flow direction as well as actin filament rearrangement, responsible for cellular mobility and adhesion. The metabolic changes mainly include increased prostacyclin synthesis, plasminogen tissular activator expression, differential regulation of adhesion molecule proto-oncogene expressions and ion transfer (K^+, Ca^{++}). Shear stresses also stimulate endothelial cell proliferation and migration.

The endothelial cell responses to mechanical stimuli involve the majority of mechanisms linked to cellular growth and metabolism. Thus, ionic channels sensitive to membrane stretching, adenylate cyclase and protein kynase C have their activity modified in response to mechanical stress. According to different works mechano-receptors exist on the cell surface and are linked to the cytoskeleton. The cytoskeleton alterations induced by shear stress would disturb cellular equilibrium and so modify the activities of receptors and trans-membrane channels. Also, gene expression is influenced by flow as well as release of endothelin 1, plasminogen tissular activator, NO, PDGF, growth factors, thrombomodulin and adhesion molecules. An initial molecular model of the regulation of gene expression under stress was recently considered. In this model the mechanical forces activate mechano-sensitive structures, and thus modify membrane receptor activity. This initial step is then applied to the second messengers like intracellular Ca^{++}, protein kinase C, cyclic AMP, cyclic GMP, etc., which in turn are disrupted. Such deep changes in the balance of the secondary messengers will then alter the activation status of the DNA binding factors. Various types of response of endothelial cells to the mechanical stress have been described and could in a simplified manner be classified into three types:

- early and transient increase (c-fos, c-jun, c-myc, PDGF, ...);
- continuous increase in mRNA expressions (t-PA, NOS, ICAM1, ...);
- two-phase regulation: increase during the first two hours followed by a continuous decrease after the 12th hour (ET-1, PDGF-β, VCAM1, ...).

5. Conclusion

It is now admitted that mechanical forces induce many key events in the physiopathology of the vascular endothelium cells. 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 of the endothelium. This concept, related to the identification of mechanoreceptors and the comparative study of the responses to mechanical stimuli of other types of cells (i.e., vascular smooth muscle cells, chondrocytes, etc.) should be of interest to understand the mechanotransdution phenomena.

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Factors influencing the transendothelial accumulation of atherogenic plasma proteins in artery walls

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1. Introduction

Atherosclerosis, which manifests itself clinically as myocardial infarction and stroke and their respective precursor disorders angina pectoris and transient ischemic attacks of the brain, is initiated by the accumulation of the plasma low density lipoprotein (LDL) in the walls of large arteries, most importantly the coronaries, carotids, and the aorta.

Abnormally high concentrations of LDL in the plasma are associated with premature clinical manifestations of atherosclerosis, indicating that the uptake of LDL increases with its plasma concentration [14]. LDL is thus a major risk factor for coronary heart disease and the other aforementioned disorders.

Another major risk factor is fibrinogen, the incidence of heart attacks and strokes increasing with its plasma concentration [12,24,46]. Atherosclerotic lesions, contain much fibrinogen as well as fibrin [38].

The mechanism by which LDL and fibrinogen and presumably also other atherogenic plasma proteins such as LP(a) pass from the blood through the endothelium into the arterial intima remains largely unknown. For LDL the process does not apparently involve the high-affinity receptors because, when they are deficient or defective as in Watanabe rabbits and in Type II familial hyperlipidemia, circulating LDL levels are abnormally high and atherogenesis is accelerated. Moreover, the uptake by arterial walls of reductively methylated LDL which does not bind to high-affinity receptors is similar to that of native LDL [23,45]. In the case of LDL there is evidence that it passes through arterial endothelia cells in cytoplasmic vesicles called caveolae [36,42]: these are identical to the pinosomes in which this lipoprotein was first observed by autoradiography [39]. This appearance has also been demonstrated with cultured endothelial cells where it depends strongly on LDL concentration [41]. The process is referred to as transcytosis, although up to the present time it has not been possible to make dynamic observations on such transendothelial movements. The evidence is essentially based on time sequences of electron micrographs and inferred for LDL from observations made with other, electron-dense particles such as ferritin after their intravascular injection [27,37]. So far, the actual mechanism where LDL and fibrinogen and presumably also Lp(a) and other circulating macromolecules are taken up by and discharged from endothelial caveola remains essentially unknown.

The purpose of the work summarized here to look for factors, other than plasma concentrations of LDL and fibrinogen, which influence their accumulation in artery wall. This process requires the passage of

the macromolecules across the arterial endothelium hence one aim of our investigations is to increase the understanding of the pathways and mechanisms whereby atherogenic plasma proteins traverse the endothelial layer.

Over recent years we have produced experimental evidence indicating that the arterial accumulation of LDL and fibrinogen is determined by structural and hormonal factors as well as by their plasma levels. Specifically, it is determined by the density of anionic site on the endothelial surface, and by the actions of endogenous and exogenous pressor agents. As the evidence has been obtained with two mammalian species, viz. rabbit and rat, these factors may operate also in man.

2. The density of anionic sites on the endothelial surface

The luminal surface of vascular endothelium is covered by a surface coat, referred to as the glycocalyx, which consists of glycoproteins, sialoconjugates, and proteoglycans and confers a net negative charge to the blood–endothelium interface. Some time ago we showed that the flux of LDL into artery walls is accelerated by the selective removal of sialic acids and/or glycosaminoglycans from arterial endothelium *in vivo*, suggesting that negatively charged components on the endothelial surface limit the movement of LDL, which is also negatively charged, from the blood into artery walls [15,21].

If the electronegative charge properties of the endothelial surface act as a barrier to plasma LDL, then a drug capable of increasing the negative charge on vascular endothelium *in vivo* should decrease the rate at which LDL is taken up by the arterial wall and slow the development of atherosclerosis. The ability of glycosaminoglycans, in particular heparin, to interact with arterial endothelium [20] suggested that it might be possible to augment the negative charge on endothelium with naturally occurring acidic molecules. This possibility was investigated with promising results. When rabbit carotid arteries were temporarily perfused with the glycosaminoglycan dermatan sulphate, the uptake of LDL by the walls of the arteries significantly decreased. Dermatan sulphate was taken up by the arterial walls *in vivo* and subsequently lost in two phases, an initial rapid phase followed by a slower exponential phase, suggesting both high and low affinity binding to the endothelial surface [22].

LDL uptake is inhibited by vessel-wall-associated dermatan sulphate, it might be expected that this could also be demonstrable as an increase in blood LDL concentration. We therefore determined the effect of intravenous infusions of dermatan sulphate on the clearance of LDL from the circulation in rabbits, and the effect of bolus intravenous injections of dermatan sulphate on the clearance of LDL in mice. The human LDL used was methylated to prevent its removal from the circulation via high-affinity receptors present in the liver as well as on vascular endothelium [44]. The clearance of methylated LDL (mLDL) from the blood was indeed significantly diminished in both rabbits and mice; variety of control experiments excluded the possibility that this effect was the result of a complex formation between dermatan sulphate and LDL. The decrease in the rate constant for the disappearance of mLDL from the blood implies a slowing down in LDL clearance via non-receptor-mediated pathways. Thus, dermatan sulphate appears to inhibit the intravascular clearance of LDL by pathways such as those involved in atherogenesis. Whether the effect was due to dermatan sulphate bound to the endothelial surface actually taken up into the vessel wall remains to be established. This work opens up the possibility for the development of anti-atherosclerotic agents carrying a negative charge and targeted specifically to vascular endothelium. The protective mechanism of such drugs would depend on reducing the arterial uptake of LDL and possibly of other atherogenic plasma proteins, including fibrinogen, rather than on lowering plasma lipoprotein levels.

3. Effects of pressor and depressor agents

Epidemiological evidence indicates that the risk of both coronary heart disease and stroke is increased multiplicatively when elevated levels of LDL and/or fibrinogen are associated with raised systolic or diastolic blood pressure [18,46]. LDL, fibrinogen, and blood pressure are independent risk factors, but the epidemiological evidence suggests some mechanistic interactions. An experimental investigation is underway to try to find out whether and how the accumulation of LDL and fibrinogen in artery walls is affected by increases in blood pressure, both in the short term and over longer periods more comparable with clinical hypertension. Observations made so far suggest that pressor agents are able to influence the accumulation *in vivo* on the two experimental species rabbit and rats suggesting that this may happen also in man.

4. Catecholamines

We found some time ago that in anaesthetized rabbits the uptake of LDL by arterial walls is accelerated by noradrenaline or adrenaline, although at blood concentrations somewhere in excess of those associated with stress in animals and man [3,35]. In these experiments the uptake of intravenously injected ^{125}I-labelled mLDL was compared in the walls of the two carotid arteries after infusing the catecholamines into the blood stream of one, the saline, into the blood stream of the other as control, the infused volumes being only 1–2 of the carotid blood flow which could, therefore, be assumed to be unaffected by the infusions. At the end of 2 or 4 h of infusion the animals were killed, the carotid arteries free of blood and excised, and their radioactivities determined. Both catecholamines at local blood concentrations of approximately 10 nmol/l caused significant increases in LDL radioactivities in the wall of the catecholamine-infused artery. Noradrenaline infused the higher concentration of 100 nmol/l also increased the LDL radioactivity of the saline-infused carotid: this increase could be accounted for by increased plasma noradrenaline concentrations in the control carotid to levels which increased LDL uptake in the noradrenaline-infused carotids.

A similar effect of adrenaline was then demonstrated in another mammal, the rat, by a very different technique in which the animals remained conscious and unrestrained [4]. Osmotic minipumps (ALZET) were implanted in rats under the skin of the neck. These pumps infused either saline or adrenaline at a rate of 0.5 μl/h to give plasma adrenaline concentrations of ca. 40 nmol/l for 6 days. The rise in plasma adrenaline concentration was associated, with a moderate rise in systolic blood pressure. Human and rat LDL prepared by sequential ultracentrifugation [2,17] was labelled with ^{125}I-labelled tyramine cellobiose (^{125}I-TC-LDL). In this labelling technique the radio-iodine tracer is attached to the protein via covalently bound tyramine cellobiose, which is trapped intracellularly and persists in artery walls while the lipoprotein itself is degraded and removed [30]. The artery wall radioactivities thus represented both degraded protein and the intact protein present at the time of measurement. For LDL, double-labelling experiments showed that after it had circulated for 24 h about 80% of aortic wall radioactivity was accounted for by degraded protein [8].

After 5 days of adrenaline infusion, ^{125}I-TC-LDL was injected intravenously with the infusion continued, and 24 h later the animals were killed with intracardiac pentobarbital and the thoracic aortae prepared for radioactivity determinations. Adrenaline infused at this concentration increased the aortic radioactivities due to rat LDL by 146% ($n = 11$) and due to human LDL by 298% ($n = 7$); both increases were highly significant.

The results suggest that the catecholamines accelerate accumulation of LDL in large arteries of rabbit and rat. If present in man, such an effect might help to account for increased manifestations of atherosclerosis in conditions associated with elevated blood catecholamine concentrations, such as the episodic increases associated with cigarette smoking [9] which help to make it a leading risk factor for coronary heart disease. It would be interesting to know whether pheochromocytomas are associated with accelerated atherosclerosis. The continuous presence of catecholamines at their physiological concentrations in the circulation may therefore promote such an accumulation process in atherognenesis.

5. Reserpine

Having shown that exogenous noradrenaline and adrenaline increase the accumulation of LDL and fibrinogen in artery walls, it seemed possible that accumulation might be decreased when endogenous catecholamines are depleted. It turns out, indeed, that reserpine, which depletes endogenous noradrenaline and adrenaline, decreases the accumulation of both exogenous and endogenous LDL in arterial walls and in the heart, although not in adrenal glands (Shafi and coworkers, unpublished observations). The experiments were done with conscious, unrestrained rats, and it was found that reserpine reduced the accumulation of administered homologous LDL and endogenous LDL labelled with tritiate cholesterol. Single injections of reserpine in different doses caused rapid lowering of plasma noradrenaline while raising plasma adrenaline, in agreement with published findings [11]. This is attributable to the rapid removal of noradrenaline from neuronal stores and the more prolonged release of adrenaline from the adrenal glands. Tissue catecholamine depletion was measured in the heart, where the lowest dose of reserpine reduced noradrenaline and adrenaline by 98% and 56% respectively and where, after higher doses, neither catecholamine remained detectable. These findings support the conclusion that atherogenesis is influenced by catecholamines. However, the effect occurred at a time when plasma noradrenaline was low but adrenaline was high. This is difficult to explain because infused adrenaline, like noradrenaline, increases LDL accumulation.

Reserpine was introduced as an antihypertensive drug during the 1940s, but its use was discontinued in Britain and other countries because of occasional serious side effects mainly depression and rarely acute heart failure. However, in at least one major European country hypertension continues to be treated with reserpine in low doses, viz. 0.1–0.25 mmol per day, in combination with a thiazide diuretic [16,43]. With this preparation the blood pressure-lowering effect becomes evident after about 2 weeks, and no serious side effects have been reported. This clinical dose of reserpine is about 40 times lower than the lowest dose of reserpine given to the experimental rats in which the antiatherogenic effect was demonstrable within 2 days. The experiments are therefore being continued by giving reserpine to rats at much lower doses for prolonged periods to find out whether the putative antiatherogenic effect remains demonstrable also under conditions more like the clinical ones.

6. Angiotensin II

The mechanism underlying the accelerating effect of the catecholamines on LDL accumulation is still uncertain. However, one possibility is that it has somehow to do with the blood-pressure-raising effects. For that reason experiments were done with another pressor agent, i.e. angiotensin II. Infused angiotensin II did not change the clearances of LDL and fibrinogen from the blood, so that effects on accumulation could not be accounted for by concentration changes in the circulating proteins. The