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Inflammation and myointimal hyperplasia. Correlation with hemodynamic forces☆

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ABSTRACT

Objectives: The aim of our study was to correlate flow dynamics and the release of inflammatory cytokines Interleukin 1, 2, 6, TNF (Tumour Necrosis Factor) alfa, both in vitro and in vivo.

Materials and methods: Endothelial cells were exposed to laminar flow (6 dyne/cm^2) in an in vitro circulatory system and the release of Interleukin 1, 2, 6 and TNF alfa was quantified by ELISA. Interleukin 1, 2, 6 and TNF alfa release was also assessed in vein grafts implanted in the arterial circulation of Lewis rats. Arterial vein grafts were explanted at different time intervals from 3 days to 12 weeks after surgery. Vein grafts implanted in the arterial circulation for 4 weeks, were re implanted in the venous circulation of syngenic Lewis rats, and the release of Interleukin 1, 2, 6 and TNF alfa, was assessed in an organ culture.

Six vein grafts (4 occluded, 2 patent) implanted in humans as femorodistal bypass were examined for the presence of myointimal hyperplasia and perigraft inflammatory cells.

Results: In vitro, endothelial cells exposed to laminar flow released an increased amount of Interleukin 1, 2, 6 and TNF alfa in comparison to endothelial cells not exposed to flow.

In experimental vein grafts implanted in the arterial circulation there was an increased release of inflammatory cytokines associated to inflammatory changes in the adventitia. Once the vein grafts were re implanted in the venous circulation, the release of these cytokines diminished, while the inflammatory changes in the adventitia regressed.

Conclusions: Increased shear stress induces release of cytokines and inflammatory changes in the adventitia. These inflammatory changes can contribute to plaque progression and to un stable plaque. These findings support the use of anti-inflammatory therapy in patients prone to develop atherosclerosis and in those who had arterial reconstructive surgery.

1. Introduction

The topographic localization of the atherosclerotic plaque suggests a potential role for hemodynamic forces in the development and distribution of atherosclerosis [14,20]. Flow separation, with the simultaneous occurrence of areas of high and low shear stress, as well as of flow reversal, have been shown to be present at the level of areas predisposed to develop the atherosclerotic plaque, like the outer wall of the carotid bulb and the origin of the internal carotid artery [23]. Several studies have correlated abnormal flow dynamics to increased production of growth factors, which could explain the proliferation of smooth muscle cells and endothelial cells [6,17,18,22,33].

The events which lead to the progression from myointimal hyperplasia to the atherosclerotic plaque are complex and involve several mechanisms [1]. Atherosclerosis has been defined as a low grade inflammatory chronic process for the presence of inflammatory cells [2,32]. A number of mediators of inflammation have been studied to try to define the pathophysiologic steps which characterised the evolution from stable to unstable plaque.

The aim of our study was to correlate different levels of flow shear stress with the release of well-known inflammatory cytokines (IL – Interleukin-1, IL2, IL6, TNF alfa) both in vitro and in vivo.

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None of the Authors has conflicts of interest to declare.

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2. Material and methods

2.1. Experimental design

2.1.1. In vitro study

Bovine endothelial cells were exposed in vitro to different levels of shear stress (0 dyne/cm^2 and 6 dyne/cm^2). Thereafter, the production of inflammatory cytokines (IL1, IL2, IL6, TNF alfa) was analysed.

2.1.2. In vivo study

2.1.2.1. First experiment. Male Lewis rats (weight 250 g) were used for the experiments. The inferior vena cava from syngenic rats was interposed at the level of the abdominal aorta. In this situation there is an abnormal proliferation of endothelial and smooth muscle cells which leads to myointimal hyperplasia of the media and inflammatory changes in the adventitia. Four weeks later, the graft was harvested ad put in organ culture and the release of IL1, IL2, IL6, TNF alfa was assessed, and compared to that of ungrafted inferior vena cava.

2.1.2.2. Second experiment. With the same schema, veins were inserted into the arterial circulation of syngenic Lewis rats and 4 weeks later explanted. Then, they were reimplanted in the venous circulation (iliac vein) of syngenic Lewis rats for 8 weeks, and then explanted At this time the inflammatory changes in the adventitia disappeared. Grafts were put in organ culture and the release of IL1, IL2, IL6 and TNF alfa was analysed.

Animal care complied with the Principles of Laboratory Animal Care as formulated by the National Society for Medical Research and Guide and Use of Laboratory Animals (US Department of Health and Human Science, National Institute of Health publication 80.23, revised 1996).

The study was approved by the Research Committee of the Laboratory and of the Department of Surgery.

2.2. In vitro study

2.2.1. Endothelial cells isolation

Endothelial cells were obtained from the thoracic aortas of calves, with collagenase digestion. The thoracic aortas were placed with the luminal surface downward in a 0.03% solution of collagenase II (Sigma Chemical CO - ST Lois-Mo). Endothelial cells were removed with a scraper. They were identified by positive immunofluorescence staining for factor VIII related antigen. A population of 1×10^6 of endothelial cells were seeded in coated polystyrene tubes with an internal diameter of 10 mm. Endothelial cells were grown in M199 with 20% fetal calf serum and antibiotics.

2.2.2. Application of shear stress to endothelial cells

A roller pump and a closed circulatory loop were used for the experiments. Dye injection showed laminar flow. The flow velocities chosen for the experiments were 0 ml/min (0 dyne cm^2), 100 ml/min (6 dyne/cm^2). The tubes with the endothelial cells were interposed in the circuit. The apparatus was kept at 37 °C in a 5% CO_2 incubator. Endothelial cells were exposed to laminar flow for different time intervals, up to 24 h, and then left without flow for an additional 24 h. The cells remained viable and attached to the tubes.

2.2.3. Assay of IL1, IL2, IL6 and TNF alfa in the conditioned media

The presence and quantity of these cytokines and growth factors in the conditioned media of endothelial cells exposed to flow and not was assessed by Enzyme-Linked Immunosorbent Assay (ELISA).

We used immunoglobulin G antibody labelled with alkaline phosphatase as previously described [13]. Bound specific antibody was quantified by optical density reading at 492 nm with a spectrophotometer.

2.3. In vivo study in animals

2.3.1. Operative procedures

Xylazine and ketamine were used for anesthesia. Animals were humanely sacrificed with an overdose of anesthetic.

In 85 animals the thoracic inferior vena cava, explanted from syngenic Lewis rats, was used as graft. It was implanted in the infrarenal abdominal aorta of syngenic Lewis rats in an end-to-end fashion: 10 0 nylon sutures were used (Ethicon Inc., Somerville, NJ) in a continuous fashion, with two starting sutures placed at 180°. Five animals died in the early postoperative period for occlusion of the graft and they were excluded from the study. Animals were humanely sacrificed at different time intervals: 3 days (10 animals); 7 days (10 animals), 4 weeks (26 animals), 6 weeks (8 animals) and 12 weeks (10 animals) from operation.

In another 16 animals, the vein graft was implanted in the arterial circulation for 4 weeks. The graft was explanted and inserted in the venous circulation (left iliac vein) of syngenic Lewis rats: the animals were humanely sacrificed 2 weeks (8 animals) and 8 weeks later (8 animals).

At harvest, the grafts were opened in the middle to assess the patency, checking the inflow and the outflow. All grafts were patent at the time of sacrifice. No antiaggregant or anticoagulation were used.

2.3.2. Histology

Transverse sections of the grafts were stained with hematoxylin and eosin. The area of the adventitia was calculated by videomorphometry (Quantimet 500). Specimens were fixed in glutaraldehyde for scanning electron microscopy at accelerating voltage (Hitachi - Japan).

2.3.3. Organ culture

The technique has been already described [14,15]. In brief, the specimens were washed with Dulbecco Modified Eagle Medium with antibiotics and placed in culture plates (Costar, Cambridge, Mass). The specimens were put in the organ culture at a pH of 7.4, at 37 °C, in a 5% CO_2 atmosphere. Aliquots of the culture media were taken at 72 h, and the concentrations of the different growth factors and cytokines was analysed by Enzyme-Linked Immunosorbent Assay (ELISA). We used immunoglobulin G antibody labelled with alkaline phosphatase as previously described. Bound specific antibody was quantified by optical density reading at 492 nm with a spectrophotometer. The specimens resulted viable after 72 h of incubation by cytofluorometry and tritiated thymidine uptake.

2.3.4. Statistical analysis

Student's *t*-test and Chi square analysis were used for comparisons. Differences were considered statistically significant at a p value of 0.05 or less.

3. Results

3.1. In vitro study

Figs. 1, 2, 3, and 4 shows the kinetics of the release of IL1, IL2, IL6 and TNF alfa. Shear stress induced the release of these cytokines and growth factors ($p < 0.01$). Each cytokine had a different kinetics to hypothesise that shear stress influenced independently the release of each single factor.

3.2. In vivo study in animals

3.2.1. Gross appearance

Veins implanted in the arterial circulation became rigid, whitish. This phenomenon was more evident at late intervals. Once, re implanted in the venous circulation, the vein grafts lost their rigidity, and become soft and flexible. The re implanted vein grafts lost their whitish appearance, and appeared pink.

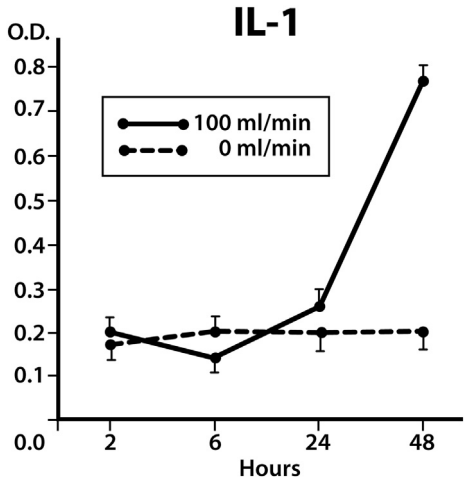

Fig. 1. Kinetics of IL-1 release by endothelial cells exposed or not to laminar flow.

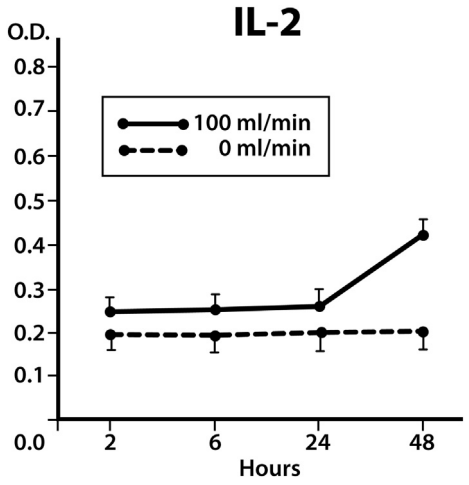

Fig. 2. Kinetics of IL-2 release by endothelial cells exposed or not to laminar flow.

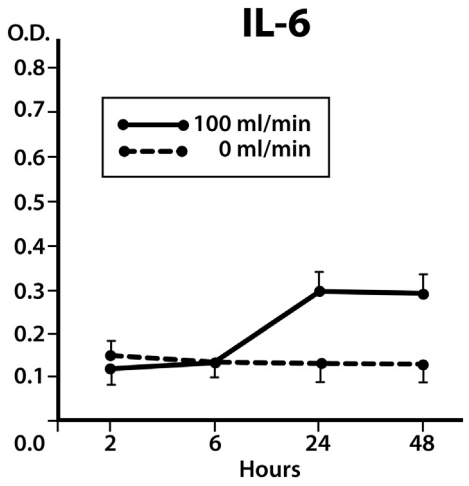

Fig. 3. Kinetics of IL-6 release by endothelial cells exposed or not to laminar flow.

3.2.2. Histology

Rarely, it was possible to observe few lymphocytes in the outer wall of the vein grafts. Collagen deposits in the outer wall of the vein graft appeared already 7 days after implantation in the arterial system. Table 1 shows the average thickness of the adventitial layer, which increased steadily after implantation in the arterial system (Figs. 5, 6).

Once re implanted in the venous system, the scattered lymphocytes in the adventitia disappeared. Fibroblasts diminished and 8 weeks after re implantation in the venous system, the adventitia of the vein graft appeared similar to that of a control ungrafted vein.

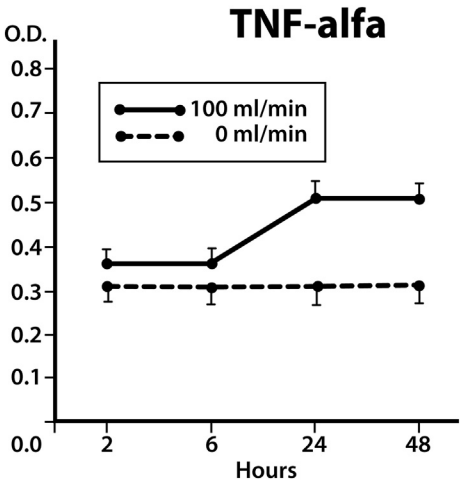

Fig. 4. Kinetics of TNF alfa release by endothelial cells exposed or not to laminar flow.

Table 1
Thickness adventitia (microns).

Control vein	10 + − 6
Arterial vein grafts	
3 days	15 + − 5
7 days	20 + − 7
4 weeks	30 + − 4
6 weeks	38 + − 5
12 weeks	40 + − 4
Reimplanted vein grafts	
2 weeks	37 + − 5
4 weeks	14 + − 5

3.2.3. Release of IL1, IL2, IL6 and TNF alfa

Tables 2 and 3 shows the release of these factors from vein grafts put in an organ culture. As soon as the vein graft was implanted in the arterial circulation the release of these factors increased and remained constantly high. Once re implanted in the venous circulation, the release of IL1, IL2 and IL6 returned to the values of a control ungrafted vein. TNF alfa returned to preoperative values only 8 weeks after re implantation in the venous system. There was a close correlation between the amount of cytokines released and the degree of inflammation and thickness of the adventia ($p < 0.01$).

4. Discussion

The epidemiological observation that biomarkers of inflammation are associated with clinical cardiovascular risk, tend to support the hypothesis that inflammation can have a detrimental effect in the development of plaque progression and instability [2,3,16,27,30,32]. Several investigators have shown that abnormal hemodynamic conditions, like flow reversal, or boundary layer separation are correlated to atherosclerotic plaque formation [14,19,20,23,24,26,28,29]. It is evident from epidemiological and experimental studies that the formation and progression of the atherosclerotic plaque is the results of multifactorial situations and events [3].

In our in vitro study, we showed that high levels of shear stress induce release of inflammatory mediators from endothelial cells. These inflammatory cytokines and TNF alfa participate in inflammation, thrombosis and many immunologic processes. IL1, IL2 and IL6 act on various cells, including B and T cells [12,15]. TNF alfa is involved in

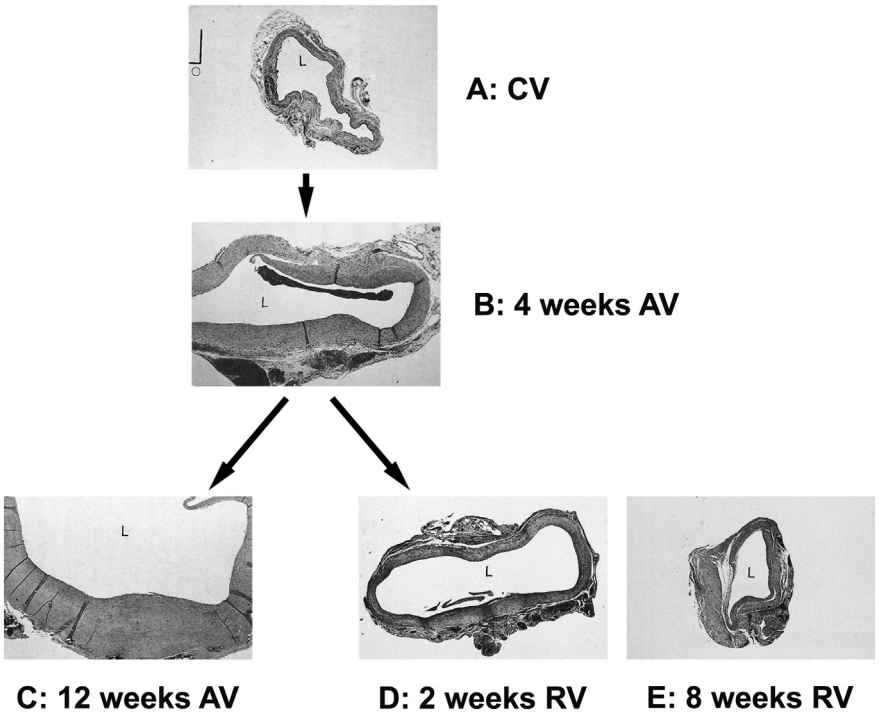

Fig. 5. Transverse sections of control vein (CV-vein before implantation in the arterial system): AV (arterial vein grafts) at different intervals and RV (vein grafts, arterialized and the re-implanted in the venous circulation) at different intervals. (Hematoxylin-Eosin × 4).

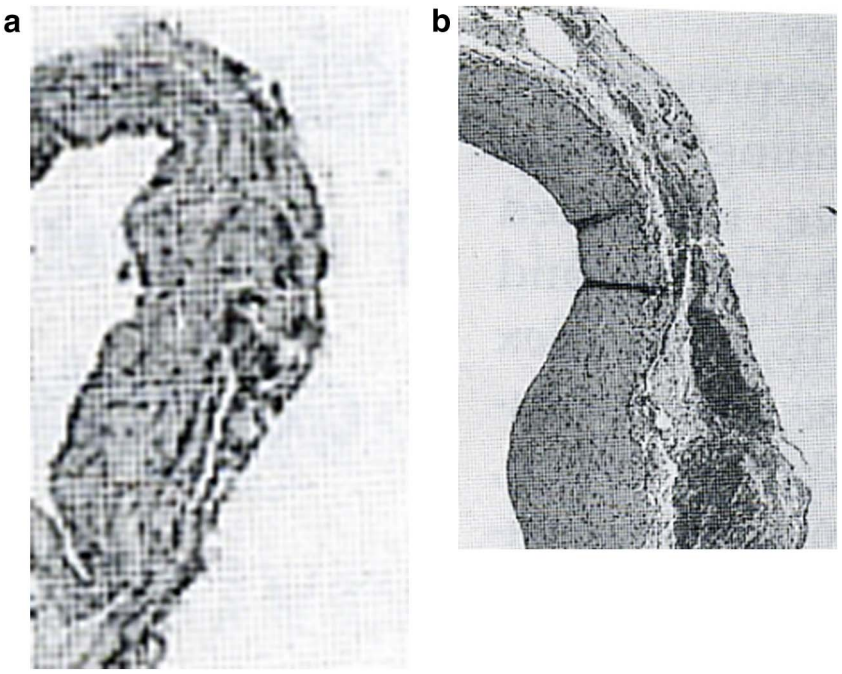

Fig. 6. Detail of periadventitial tissue in a transverse section of a control vein (before implantation) (A-left) and of vein graft implanted into the arterial circulation form 4 weeks (B-right) (Hematoxylin-Eosin × 16).

many aspects of inflammation, including macrophages activation [5,7,8]. This increased production of IL1, IL2, IL6 and TNF alfa can explain some of the inflammatory changes associated with myointimal hyperplasia formation.

Conceptually, these inflammatory changes can lead to un stability of the atherosclerotic plaque, which can ulcerate with lumen thrombus formation and distal embolization, through the stimulation of new vessel formation, inside and around the plaque. This new vessels,

Table 2
Growth factors and cytokines release in the organ culture by arterial vein grafts (av).

ng/cm^2	Control vein	AV 3 days	AV 7 days	AV 4 weeks	AV 6 weeks	AV 12 weeks
IL1	0.2 + − 0.05	3.8 + − 1.0	3.6 + − 0.9	3.8 + − 0.9	3.8 + − 0.8	4.9 + − 2.0
IL2	0.2 + − 0.03	2.5 + − 0.9	4,0 + − 0.8	4.2 + − 0.3	4.0 + − 0.3	4,3 + − 0.3
IL6	0.01 + − 0.01	3.0 + − 0.3	3.9 + − 0.2	4.0 + − 0.4	3.9 + − 0.2	4.0 + − 0.2
TNF alfa	24 + − 2.8	27 + − 3	33 + − 3	50 + − 6	219 + − 21	200 + − 19

Table 3
Cytokines release in the organ culture by re implanted vein grafts (rv).

ng/cm^2	Control vein	RV 2 weeks	RV 8 weeks
IL1	0.2 + − 0.05	0.5 + − 011	0.3 + 0.1
IL2	0.2 + − 0–03	0.4 + − 0.1	0.3 + − 0.05
IL6	0.01 + − 0.01	0.6 + − 0.05	0.2 + − 0.1
TNF alfa	24 + − 2.8	220 + − 15	23 + − 2.1

subjected to abnormal flow dynamics can disrupt with haemorrhage which contribute to plaque progression and un stability [4,10,13,16,21].

The correlation between high levels of shear stress and release of high quantities of cytokines and TNF alfa was confirmed in our study in experimental vein grafts. The high production IL1, IL2, IL6 and TNF alfa was correlated with the simultaneous presence of inflammatory changes, such as increased fibroblasts proliferation and collagen deposition.

Once the vein graft was re implanted in the venous circulation, this inflammatory changes disappeared almost completely in 8 weeks; at the same time IL1, IL2, IL6 and TNF alfa release returned to preoperative value. These in vivo findings demonstrated a direct correlation between increased levels of shear stress and inflammatory changes, through high release of IL1, IL2, IL6 and TNF alfa.

There were some differences between the kinetics of in vitro and in vivo release of inflammatory cytokines. It is difficult to comment on these differences. We cannot exclude that in vivo, other factors, able to partially reduce the release of inflammatory cytokines, are produced simultaneously by circulating cells [1,2].

The correlation between abnormal hemodynamic forces and atherosclerotic plaque formation and progression, through an increased release of mediators of inflammation, can be applied to both the primary atherosclerotic plaque and to the formation of myointimal hyperplasia and atherosclerosis which are the most common cause of occlusion of arterial reconstructions, including, endovascular stenting [11,25].

In our study normal endothelial cells from healthy animals were tested. Caution should be taken in extrapolating the findings of our study to the aging, dysfunctional endothelial layer of the artery with atherosclerosis. We can assume that in the initial process of atherosclerosis formation, and in specific pathological conditions, altered flow dynamics with the simultaneous occurrence of high and low shear stress, and therefore turbulence and possibility of flow reversal, can determine a chronic scenario, with the final result of a steady increased release of inflammatory cytokines.

The results of our findings, and the potential detrimental effect of the observed inflammatory changes can have important clinical implications. High levels of anti-inflammatory agents can have a major therapeutic role to prevent myointimal hyperplasia formation and progression. This therapeutic approach can be extended to patients who had undergone arterial open or endovascular surgery.

Several studies have shown improved results after open and endovascular surgery, when patients had double and even triple anti platelet therapy [9,31,34]. This improved results have been attributed to their action on platelets. Is not possible that the positive effects of this aggressive anti platelet therapy, are due mainly to their associated anti-inflammatory action?

5. Conclusions

On the basis of the results of our study, we can conclude that increased release of inflammatory cytokines is associated to myointimal hyperplasia, which is the first “momentum” in atherosclerotic plaque formation. Abnormal flow dynamics, with the simultaneous presence of high and low levels of shear stress, and flow reversal, predispose to smooth and endothelial cells proliferation which are the basis of myointimal hyperplasia formation. The same abnormal flow dynamics lead to high production of inflammatory mediators, which could lead to plaque unstable.

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