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Leucocyte recruitment in rupture prone regions of lipid-rich plaques: a prominent role for neovascularization?

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Abstract

Objective: Microvessels in atherosclerotic plaques provide an alternative pathway for the recruitment of leucocytes in the lesions. The present study was designed to investigate the potential role of these microvessels in creating vulnerable sites in atherosclerotic plaques. **Methods:** Thirty-four atherosclerotic plaques were obtained from 25 patients undergoing carotid endartherectomy ($n=16$), femoral endartherectomy ($n=6$) and aortic surgery ($n=12$). Plaques were histologically classified as either lipid-rich (rupture prone, $n=21$) or fibrous (stable, $n=13$). Serial cryostat sections were immunohistochemically investigated using monoclonal antibodies against endothelial cells (ULEX-E and F-VIII), vascular endothelial growth factor (VEGF), endothelial adhesion molecules (ICAM-1, VCAM-1, E-Selectin, CD40) and inflammatory cells (macrophages (CD68) and T lymphocytes (CD3). **Results:** The microvessel density in lipid-rich plaques was significantly increased as compared to fibrous plaques. Most of these vessels were located in the shoulder-region of the plaque and at the base of the atheroma. Microvessels in lipid-rich plaques also expressed increased levels of ICAM-1, VCAM-1, E-Selectin and CD40. Moreover, inflammation was most abundantly present in the proximity of microvessels. VEGF was only observed on vessels and mononuclear cells in lipid-rich plaques, suggesting that this factor may play a role in microvessels formation. **Conclusions:** Neovascularisation and expression of adhesion molecules by microvessels at sites of vulnerable lipid-rich plaques may sustain the influx of inflammatory cells and hence, could contribute to plaque destabilization.

Keywords: Atherosclerosis; Inflammation; Adhesion molecules; Plaque stability

1. Introduction

The recruitment of inflammatory cells in atherosclerotic lesions is a constitutive phenomenon throughout the process of lesion initiation and plaque growth. In addition, inflammation appears to play a role in the process of plaque disruption which underlies the acute thrombotic complications of atherosclerosis [1,2]. Plaque rupture occurs in many instances at the shoulder region of the plaque and is always associated with the presence of inflammatory cells at the immediate site of rupture [1,2]. In general, large densities of inflammatory cells in unruptured plaques are present in the shoulder parts, in the cap, or both. In many instances and, particularly in advanced plaques with a complex architecture, they tend to accumulate focally within plaques. Presently there is no explanation for these focal accumulations in advanced plaques.

The presence of microvessels in atherosclerotic plaques has been known for over a century [3,4]. Only recently it was shown that the endothelial cells of microvessels in atherosclerotic plaques express adhesion molecules ICAM-1, VCAM-1 and E-selectin. This observation has introduced the concept that these microvessels may contribute to the recruitment of leucocytes in the plaques [5]. Thus far, however, nothing is known about the potential clinical importance of these microvessels in terms of contributing to plaque instability. For this reason we have analyzed

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microvessels in fibrous plaques, generally believed to be clinically safe, and lipid-rich plaques, widely considered to be at risk.

2. Methods

2.1. Specimens

Vessel wall fragments were collected during vascular surgery procedures and immediately frozen in liquid nitrogen. The investigation conforms with the principles outlined in the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Academic Medical Hospital. The study included 34 plaques from 25 patients (15 male, 10 female, mean age 65.2±10.1 years). The specimens were collected from the aorta (12), the carotid arteries (16), and the femoral arteries (6). From each vessel, 16 serial sections were investigated. Plaque morphology was evaluated using hematoxylin eosin stains; specimens were classified as either fibrous, in which the plaque consisted predominantly of fibrocellular and/or fibrosclerotic tissue with or without small extracellular lipid cores, or lipid-rich, characterized by a large lipid core (more than 40% of the intimal surface area) and thin fibrous cap [6]. Ruptured atherosclerotic plaques were not encountered in this series.

2.2. Immunohistochemistry

Serial cryostat sections (5 μm) were cut, air dried and stored at −20°C. Prior to immunostaining, sections were fixed in cold acetone; the antibodies used are documented in Table 1. Immunohistochemical stains were performed using either a streptavidin–HRP, a streptavidin–biotin complex technique, or the enhanced polymer one-step staining (EPOS) system from Dako, as described previously [7,8]. In short, sections were successively incubated with predetermined optimal dilutions of the primary antibodies, 0.3% H_2O_2 and 0.1% NaN_3 in PBS (blocking of endogenous peroxidase), biotinylated rabbit anti-mouse immunoglobulins (Ram-Bio, Dakopatts, Glostrup, Denmark). Subsequently, either HRP conjugated streptavidin or the streptavidin–biotin–HRP (both from Dakopatts). Enzyme activities were detected using 3.3 amino-9-ethyl carbazole (AEC) as chromogen and H_2O_2 as substrate. Stains with VEGF were performed using the EPOS system [8]. After blocking endogenous peroxidase activity, sections were incubated with the VEGF antibody, followed by HRP-conjugated goat anti-mouse-EnVision+ (Dako). Finally, enzyme activity was visualized using AEC. Incubations of sections where the specific primary antibody had been omitted or had been replaced by isotype-matched non-binding antibodies served as negative controls.

2.3. Quantification of immunohistochemical results

All sections were studied independently by two different observers. Surface areas of plaques were measured using TIM image software (Difa, Breda, Netherlands) [9]. The number of microvessels in each plaque was counted and expressed as the number of vessels per mm^2 (microvessel density). The number of microvessels positive for a particular adhesion molecule was counted and divided by

Table 1
List of monoclonal antibodies used in this study

MAb (clone)	CD	Distribution	Detection method[a]	Supplier[b]
Leu-4	CD3	Pan T cells	Strep–HRP	B&D
CLB 14G7	CD40	Human CD40	sABC	CLB
M3	CD40	Human CD40	sABC	Genzyme
	CD40L	Ligand of hu-CD40	sABC	ancell
	CD40L	Ligand of hu-CD40	sABC	Calbiochem
ICAM-1	CD54	Broad		R&D
BBA3				
PG-M1	CD68	Macrophages	sABC	Dako
VCAM-1	CD106	Activated endothelial cells	Strep–HRP	R&D
E-Selectin (ena-1)	CD62E	Activated endothelial cells	Strep–HRP	Monosan
F-VIII	−	Endothelial cells	Strep–HRP	Dako
SMA	−	Smooth muscle actin	Strep–HRP	Dako
(IA4)				
Ulex E	−	Endothelial cells	RαUEA/HRP	Dako
(UEA)				
VEGF	−	Vascular endothelial growth factor	EPOS	R&D

[a] sABC: streptavidin–biotin complex; strep–HRP: streptavidin–HRP; EPOS: enhanced polymer one-step technique; RaUEA/HRP: HRP conjugated rabbit immunoglobulins anti Ulex-E agglutinin.
[b] Ancell, Bayport, MN, USA; B&D: Becton Dickinson, San Jose, CA, USA; Calbiochem, San Diego, CA, USA; CLB: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands; Dako: Dakopatts, Glostrup, Denmark; Genzyme, Cambridge, MA, USA, Monosan, Uden, Netherlands, R&D Systems, Abingdon, UK.

the total number of microvessels present in the plaque (judged from the adjacent section stained with ULEX-E) and expressed as a percentage.

Since counting the absolute number of inflammatory cells in plaques was impossible and area quantification did not cater for the specific location of inflammatory cells in relation to the microvessels, we have relied upon a semiquantitative approach. Thus, inflammatory infiltrates were scored in three grades of severity as follows: (1) less than five inflammatory cells either around microvessels or without vascular structures (2) focal clusters of inflammatory cells around vascular structures (3) diffuse inflammatory infiltration of areas with microvessels.

3. Results

Of the 34 atherosclerotic plaques, 21 were classified as lipid-rich and 13 as fibrous.

3.1. Microvessels

In 19 out of 21 lipid-rich plaques microvessels were observed (Table 2). These vessels were found in the shoulder regions of plaques, or deeply located in the intima beneath the atheromatous core. In 5 out of 13 fibrous plaques microvessels were present, which is significantly less compared to lipid-rich plaques ($P<0.005$, χ^2 test). Microvessel density in lipid-rich plaques was found to be significantly higher as compared to fibrous plaques ($P<0.05$, Student's *t*-test). Representative examples of a lipid-rich and a fibrous plaque, stained with a monoclonal antibody against endothelial cells are illustrated in Fig. 1A and Fig. 2A, respectively.

3.2. Inflammation

In 6 out of 13 fibrous plaques inflammation was absent (Table 2), but in the remaining 7 fibrous plaques focal aggregates of inflammatory cells ($CD68^+$ or $CD3^+$) were observed, frequently in association with microvessels. On the other hand, in only two lipid-rich plaques inflammatory cells were absent; these plaques were also devoid of microvessels. In all other sections of lipid-rich plaques focal aggregates (grade 2) or diffuse infiltration (grade 3) with inflammatory cells was observed. They were found mostly in the shoulder region of the plaque or beneath the atheromatous core near microvessels. Occasionally, inflammatory cells were observed inside microvessels. Differences in the extent of microvascularization and inflammation between fibrous plaques and lipid-rich plaques are given in Table 2. Representative examples of fibrous and lipid-rich plaques, stained for macrophages and lymphocytes, are illustrated in Fig. 1F–G and Fig. 2F–G, respectively.

3.3. Adhesion molecules and microvessels

In fibrous plaques ICAM-1, VCAM-1, E-Selectin or CD40 were observed only occasionally on microvessels, if present. Microvessels in lipid-rich plaques expressed significantly increased levels of E-Selectin and CD40 (Table 2). ICAM-1 and VCAM-1 were expressed not only by endothelial cells, but by many other cells also, scattered throughout the plaque as well as perivascular in location; this phenomenon made accurate quantification, particularly of small capillary vessels, impossible. However, strong expression of ICAM-1 and VCAM-1 on numerous dilated microvessels clearly showed increased expression of these molecules by endothelial cells in lipid-rich plaques (see Fig. 1B and C, Fig. 2B and C). CD40 was expressed also by other cells in the lipid-rich plaque, including macrophages and smooth muscle cells (SMC).

CD40L was found to be expressed by mononuclear cells and only in lipid-rich plaques. However, their number was relatively low. CD40L-positive cells were encountered always in small clusters and near the endothelium, either luminal or neovascular. Representative examples of lipid-rich and fibrous plaques, stained with adhesion molecules, are illustrated in Fig. 1B–E and Fig. 2B–E.

3.4. Vascular endothelial growth factor

From each of the 25 patients included in the study only one section was studied for the presence of VEGF. Fifteen

Table 2
Presence of microvessels, inflammation, and endothelial adhesion molecule expression in lipid-rich and fibrous atherosclerotic plaques

Plaques	Microvessels	Microvessel-density (mm^2)	Inflammation[d]			E-selectin	CD40	VEGF[a]
			(1)	(2)	(3)	(%)	(%)	
Fibrous	5/13	0.6±0.7	6	7	0	4	1	0/10
Lipid-rich	19/21[b]	2.1±2.6[c]	2	5	14	41[c]	27[c]	9/15[b]

[a] Twenty-five atherosclerotic plaques studied; one from each patient.
[b] $P<0.005$, as compared to fibrous plaques, χ^2 test.
[c] $P<0.005$, as compared to fibrous plaques, Student's *t* test.
[d] See Section 2 for inflammatory score.

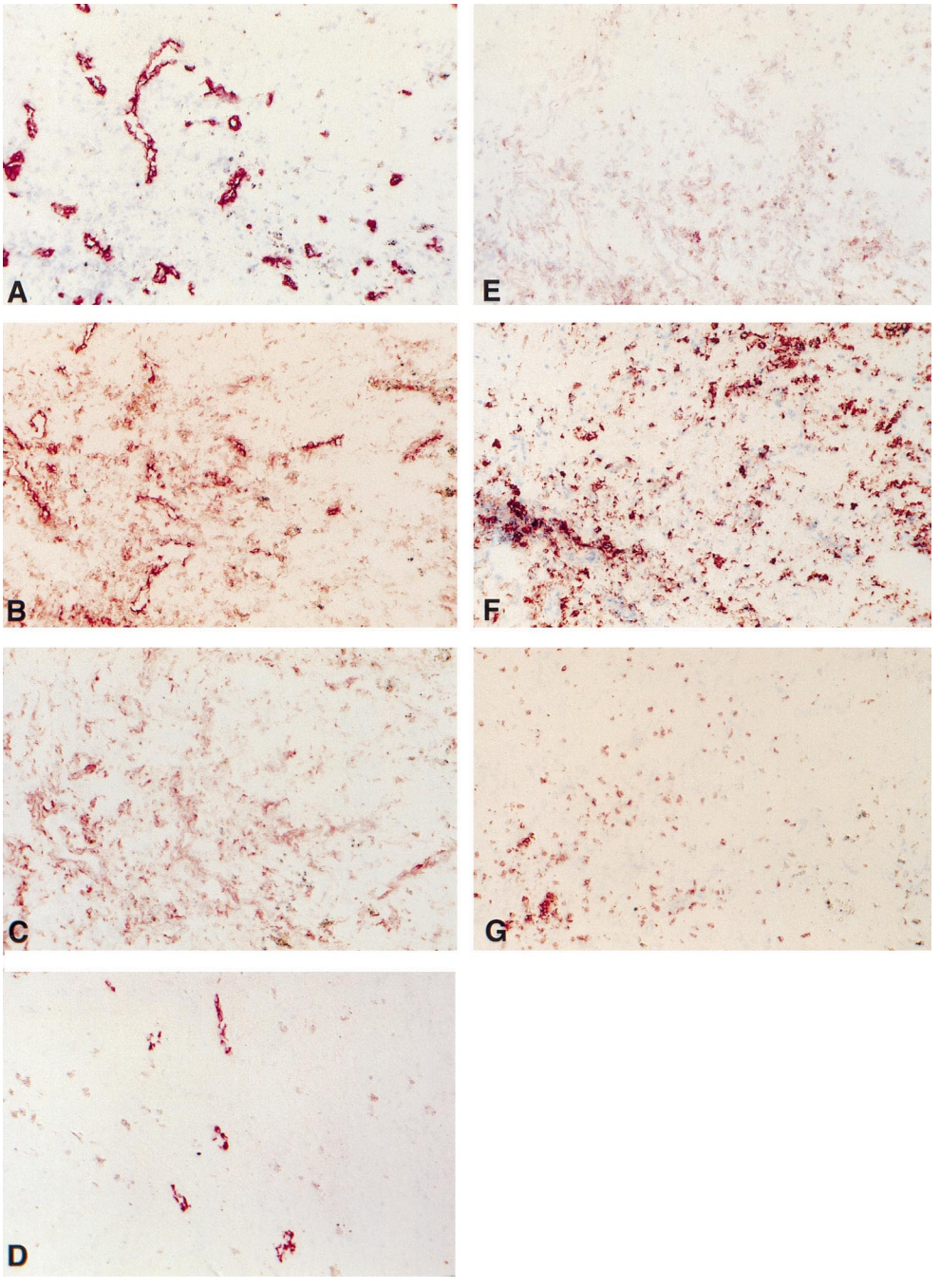

Fig. 1. Composite micrograph of adjacent sections of a lipid-rich plaque. (A) ULEX-E. Note the presence of microvessels, of which several are dilated; (B) ICAM-1, besides many ICAM-1 positive microvessels several other cells (mononuclear cells, SMC) are ICAM-1 positive; (C) VCAM-1, note positive staining by microvesels and other cell types; (D) E-selectin, several microvessels strongly express this adhesion receptor; (E) CD40, (although weak), expressed by neovessels and other cells; (F) CD68, note the high macrophage density in this type of lesion; (G) CD3, many T cell are present.

lipid-rich and ten fibrous plaques were encountered. In none of the fibrous plaques was VEGF immunoreactivity observed. On the other hand, in 9 out of 15 sections from lipid-rich plaques, immunoreactivity was observed in at least one or more microvessels, although the overall number of VEGF-positive vessels was relatively low. Moreover, VEGF positivity was found in inflammatory (mononuclear) cells.

4. Discussion

This paper reports that lipid-rich plaques contain significantly increased numbers of microvessels with a significantly higher number of microvessels expressing adhesion molecules compared to fibrous plaques. In addition, lipid-rich plaques contain increased numbers of inflammatory cells around the microvessels.

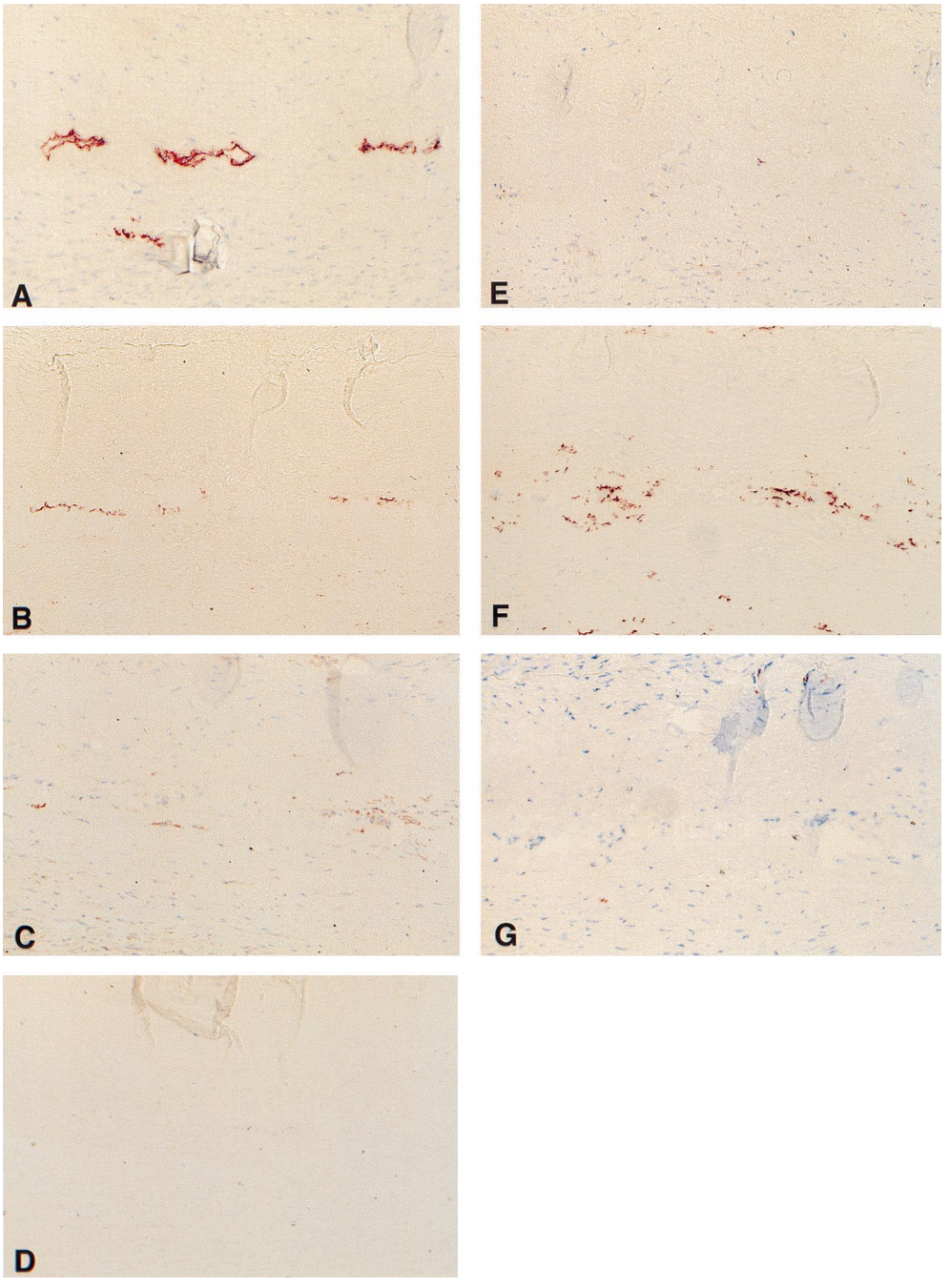

Fig. 2. Composite micrographs of adjacent sections of a fibrous plaque. (A) ULEX-E staining. Note the low density of neovessels; (B) ICAM-1, only the microvessel and some scattered cells are positive; (C) VCAM-1: a faint staining of the microvessel; (D) E-selectin, negative; (E) CD40, negative; (F) $CD68^{+}$, some scattered macrophages are present around microvessel; (G) CD3, T cells are almost absent.

The fact that atherosclerotic lesions contain microvessels has been known for more than a century [3,4]. The observation that microvessels in atherosclerotic lesions are often associated with inflammatory cells suggests that they may serve as an important gateway for leukocyte entry into the plaque [5]. However, expression of appropriate adhesion molecules and their ligands by endothelial cells and leucocytes are essential for this process. Increased expression of adhesion molecules by endothelial cells has been reported in many different studies, but almost all these studies focused on luminal endothelial cells [10,11], and some studies also included adventitial endothelial cells [12], whereas in most of these studies microvessels were neglected. Only recently O'Brien et al. examined the expression of adhesion molecules on microvessels in atherosclerotic lesions [5]. In that study a correlation was found between the presence of microvessels, expression of adhesion molecules and the presence of inflammatory cells

in atherosclerotic lesions [5]. The present study confirmed our earlier observations that these cells are abundantly present in lipid-rich plaques [13]. Moreover, this study shows that many inflammatory cells and microvessels are present in lipid-rich, but not in fibrous plaques, and that expression of adhesion molecules is increased, particularly in lipid-rich plaques. The increased expression of ICAM-1, VCAM-1, E-selectin and CD40 is most likely the result of cytokines (e.g. IL-1, TNF-α, IFN-γ) produced by T cells and macrophages, present in the lipid-rich atherosclerotic plaques. However, minimal modified lipoproteins are also able to induce or upregulate adhesion molecule expression on endothelial cells [14], suggesting that lipids may also contribute to the regulation of adhesion molecule expression on microvessels in lipid-rich plaques.

Microvessels, expressing high levels of adhesion molecules and surrounded by inflammatory cells were usually found in the shoulder regions of the plaques. The shoulder regions of atherosclerotic plaques are the most vulnerable sites and, indeed, plaque rupture often occurs in these particular regions [1,2]. Since inflammatory cells are thought to play an important role in plaque destabilization, one may consider the role of microvessels important in this context. Local activation of macrophages and T lymphocytes may lead to the secretion of growth factors or pro-inflammatory cytokines with angiogenic properties; see for review [11]. In this setting it is of interest that VEGF could be one of the factors involved. VEGF is an endothelial specific growth and chemotactic factor, which can be synthesized by various cell types including macrophages, T lymphocytes and mast cells [15]. VEGF protein and mRNA have been demonstrated by others in SMC and T lymphocytes of atherosclerotic atherectomy specimens [16]. We observed VEGF immunopositivity in most lipid-rich plaques. This suggests that VEGF, at least in part, could be responsible for the formation of microvessels in atherosclerotic lesions. The factors responsible for the induction of VEGF production in plaques are not known. One possibility is hypoxia, a common phenomenon in several pathological conditions like wound healing, tumors and inflammation [13]. There are more cytokines that are able to induce angiogenesis, e.g. tumor necrosis factor-a, interleukin (IL)-1, IL-2 and IL-8 [15,17]. It is interesting to note that increased levels of IL-8 are produced by lipid-laden macrophages, and is indeed present in human atherosclerotic plaques [18]. The net effect may result in neovascularization of the intima, and upregulation of adhesion molecules by the endothelial cells lining these microvessels. As a result, microvessels may provide a source for a more or less continuous recruitment of new leukocytes and, hence, ongoing inflammation. By the same token, however, cytokines may contribute to local degradation of extracellular matrix proteins, either by direct effects of cytokines such as IFN-γ, or by (CD40-CD40L) interactions between SMC and activated T lymphocytes, respectively [19,20]. Moreover, IFN-γ is known to inhibit the proliferation of SMC, suggesting that infiltration of atherosclerotic plaques with T lymphocytes may lead to a reduction in the number of SMC [21]. By this mechanisms, the local inflammatory process may contribute to weakening of the fibrous cap, eventually leading to plaque rupture.

We also investigated the distribution of the ligand of CD40, CD40L in fibrous and lipid-rich atherosclerotic plaques. However, in our specimens we only occasionally observed CD40L-positive lymphocytes in lipid-rich plaques. The fact that CD40L is only expressed by activated T lymphocytes, indicates that only few T cells in our specimens were activated.

In conclusion, the exuberant formation of microvessels, likely to be induced by the inflammatory process itself, may result in continuous recruitment of inflammatory cells, and thus may provide an understanding of the smoldering inflammation, which is so characteristic of atherosclerotic lesions, as well as the progression of unstable conditions and eventually the complicated plaque.

4.1. Limitations of the study

Ruptured atherosclerotic plaques were not encountered amidst the vessel wall fragments removed during vascular surgery. We could have turned to autopsy material but we preferred to use arterial fragments immediately frozen after removal from the body. Despite the fact that no ruptured plaques were studied we do think that the results strongly suggest a relationship between plaque vulnerability and the presence of microvessels. Lipid-rich plaques, widely accepted as the vulnerable type of plaque morphology, contained a significant increase in microvessel density and associated numbers of inflammatory cells, compared to ‘stable’ fibrous plaques and, moreover, these phenomena also showed preference for the shoulder regions of plaques — areas known to be the most vulnerable when it comes to plaque rupture.

In the present study the distinction between lipid-rich and fibrous plaques was based on an arbitrary cut-off point of 40% (see Section 2), as described by Davies [6]. However, the amount of fat present may vary considerably and, in fact, produces a spectrum of morphologies with lipid-rich and fibrous plaques as the extremes [13]. It would be of interest, therefore, to quantify the lipid core in relation to the microvessel density and inflammatory infiltrate.

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