

## Overexpression of hypoxia/inflammatory markers in atherosclerotic carotid plaques

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## 1. ABSTRACT

Hypoxia, angiogenesis and inflammation leads to plaque progression and remodelling and may significantly contribute towards plaque rupture and subsequent cerebrovascular events. Our aim was to study, markers of hypoxia and inflammation previously identified by microarray analysis, in atherosclerotic carotid arteries with low to moderate stenosis. We hoped to describe different cellular populations expressing the studied markers. The location of selected inflammatory molecules obtained as vascular transplants from organ donors were analysed by immunohistochemistry with monoclonal and polyclonal antibodies. Paraffin-embedded sections were cut and probed with antibodies recognizing active B and T-lymphocytes (CD30), hypoxia-inducible factor-1alpha, endoglin (CD105), Interleukin-6 and C-reactive protein. We observed a notable overexpression of HIF-1alpha in inflammatory and hypoxic areas of carotid arteries in all types of lesions from type II-V taken from the patients with carotid stenosis <50%. This suggests that HIF-1 $\alpha$  may have a putative role in atherosclerosis progression and angiogenesis. Dynamic changes in the non-occluding plaques may explain some of the clinical events in patients with low to moderate carotid stenosis.

## 2. INTRODUCTION

Carotid artery atherosclerosis is the primary cause of ischaemic stroke, which is a leading cause of death and disability in the Western world. The development of symptoms follows conversion of stable plaques to unstable ones, concomitant with the appearance of intraplaque haemorrhage, fibrous cap thinning, and infiltration with macrophages and lymphocytes T, as well as surface ulceration, rupture and thrombosis (1, 2). It is well established that the later processes occurs in the end-stage carotid plaques with significant vessels stenosis i.e. over 70 %, with indication for carotid endarterectomy. However, recent data suggests that low-to-moderate plaques which do not provoke carotid stenosis of more than 50% undergo active metabolic and inflammatory changes leading to their instability (3). When atherosclerotic lesion develops, the arterial wall thickness increases and oxygen diffusion capacity is impaired. At the same time, oxygen consumption is augmented, and an energy imbalance may occur. These local metabolic disturbances result in progression of atherosclerosis, plaque growth and remodelling, with the formation of a necrotic core. In animal models, hypoxia-mediated injury has been demonstrated in atherosclerotic plaques (4). The major transcription factor that is involved in the adaptive response

to hypoxia is hypoxia inducible factor (HIF-1). It consists of HIF-1 $\alpha$  and beta subunits. HIF-1 $\alpha$  has been identified as a basic helix-loop-helix-PAS (bHLH-PAS) family member, which is essential in the oxygen-dependent regulation of these genes (5). HIF-1 $\alpha$  is a key transcription factor for the hypoxic induction of angiogenic factors (6). It is associated with advanced inflammatory plaques rich in extracellular lipids. Furthermore, non-hypoxic activation of immune and inflammatory responses may be involved in plaque HIF-1 $\alpha$  over expression (7).

Increasing evidence suggests that inflammation is essential in the pathogenesis of atherosclerosis. In prospective studies, serum levels of inflammatory mediators such as Interleukine-6 (IL-6) and C-reactive protein (CRP) have been shown to predict future vascular events (8, 9). CRP is an effector molecule able to induce and promote atherothrombosis. The presence of CRP in atherosclerotic plaques may reflect local production or infiltration from circulating CRP increased in general inflammatory responses (1). In previous studies we demonstrated that CRP is located in cell-rich areas, vascular smooth muscle cells (VSMC), newly formed blood vessels and inflammatory cells in "active" plaques (1). The pro-inflammatory cytokine IL-6 is the primary inducer for CRP release from the liver and the only substance known to induce synthesis of all acute-phase proteins (10). Further, IL-6 acts locally to enhance recruitment of monocytes and T cells and acts systemically to induce a pro-inflammatory and pro-thrombotic state (11).

Angiogenesis is a recognized feature of the atherogenic process, with intimal neovascularization arising most frequently from the dense network of vessels in the adventitia, adjacent to a plaque, rather than from the main artery lumen (12). However, the possibility exists that some of the newly formed blood vessels are derived from circulating progenitor cells (13). It has been suggested that these new blood vessels are inherently weak and therefore responsible for development of intraplaque haemorrhage, sudden increase in plaque volume and the development of plaque instability (14). CD105 (endoglin) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells (15, 16). CD105 expression induced by hypoxia has been reported to be due to HIF-1 $\alpha$ , which directly binds to the hypoxia response element in the CD105 promoter (17). CD105 is almost universally expressed in microvessels within the atheroma and is therefore a better vascular marker than CD31 and TGF- $\beta$ 1 to assess neovascularisation in atherosclerotic plaques (18).

Atherosclerotic progression represents a chronic inflammatory reaction involving the participation of the innate immune system and is modulated by the adaptive immune system (19). The main cell types in human atherosclerotic lesions are macrophages, VSMC, and T lymphocytes (20). This suggests that a cellular immune response takes place within the lesion. Oxidized lipoproteins, heat shock proteins, and micro-organisms have been implicated as candidate antigens (21). T- and B-lymphocytes are activated during atherogenesis, primarily through cytokine

secretion and immunoglobulin production. T-cells have the capacity to modulate the development of atherosclerosis, and their influence is linked to the secreted pro-inflammatory T-helper 1 (Th1) or anti-inflammatory Th2 cytokines (22). The surface antigen CD30 is a 120-kD membrane bound glycoprotein belonging to the tumor necrosis factor (TNF) receptor super-family (23). CD30 expression is found on activated B- and T-lymphocytes (24).

We studied the presence and location of hypoxia/inflammatory markers in atherosclerotic carotid arteries with low to moderate stenosis (less than 50% by EcoDoppler imaging). We aimed to describe different cellular populations expressing the studied markers.

### 3. MATERIALS AND METHODS

#### 3.1. Carotid specimens and anatomo-pathology

We included 15 carotid arteries obtained as vascular transplants from organ donors or post-mortem autopsies. Patient's basic clinical data, vascular risk factors and immediate cause of death are presented in Table 1. Carotid arteries, including the common carotid artery, and a large portion of internal and external carotids were excised by a vascular surgeon as a part of a standard procedure for organ transplantation. There was no time delay as the other organs were removed simultaneously. The whole block of carotids was removed, immediately rinsed in sterile 0.9% saline, snap-frozen in liquid nitrogen and stored at -80°C. Later, the carotid bifurcation area was re-cut, fixed for 24 hours in buffered formalin, briefly decalcified to remove excess calcium and embedded in paraffin. Sections (5  $\mu$ m) were cut on microtome. Plaque morphology was assessed by haematoxylin and eosin-stained sections. Carotid plaques were classified according to the American Heart Association (AHA) with some modifications (25). The study was approved by the local ethical committee in accordance with institutional guidelines and family's written informed consent was obtained.

#### 3.2. Immunohistochemical analysis

Paraffin-processed sections (5  $\mu$ m) were deparaffinized in xylene and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 10% hydrogen peroxide in methanol (30 minutes at room temperature, RT) to remove endogenous peroxidase activity. Sections were blocked with respective antisera (30 minutes at RT), stained with antibodies recognizing HIF-1 $\alpha$  (1:2000, mouse monoclonal antibody, Abcam, Cambridge, UK), mouse monoclonal anti-human CD30 recognizing active B and T-lymphocytes (1:20, Dako Cytomation, Glostrup, Denmark), anti-CRP (1:40, sheep polyclonal antibody, R&D Systems, Abingdon, Oxford, UK), anti-CD105 (1:50, goat polyclonal antibody, R&D Systems, Abingdon, Oxford, UK) and anti-IL-6 (1:400, rabbit polyclonal antibody, Abcam, Cambridge, UK) and were then incubated overnight at 4°C. For HIF-1 $\alpha$  and CD30 markers, antigen retrieval was carried out. After washing in PBS, they were incubated with the appropriate secondary antibodies (1:200) for 1 hour at RT. After rinsing in PBS, standard Vectastain

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**Table 1.** Clinical and carotid anatomic-pathological characteristics of studied patients

Subjects	Age/Sex	Vascular Risk Factors	Cause of Death	Carotid Plaque AHA classification
1	74/male	DM <sup>2</sup> II, alcohol abuse	Sudden respiratory death	V
2	69/male	Smoking, alcohol abuse	Pulmonary neoplasm	III
3	76/male	none	Vesical neoplasia	V
4	74/male	HTA <sup>1</sup> , DLP <sup>3</sup> , stroke	Multiorgan dysfunction	V
5	76/male	HTA <sup>1</sup> , DM <sup>2</sup> II, DLP <sup>3</sup>	Sudden Cardiac death	IV
6	72/male	Smoking, alcohol abuse, HTA <sup>1</sup> , Obesity	Aortic rupture	V
7	70/male	DM <sup>2</sup> II, smoking, alcohol abuse,	Sudden cardiac death	V
8	82/male	Smoking, HTA <sup>1</sup> ,	Pulmonary neoplasm	II/III
9	63/female	Stroke, CAD <sup>4</sup>	stroke	II
10	76/female	None	Fatal MI <sup>5</sup>	I/II
11	55/male	HTA <sup>1</sup> , DLP <sup>3</sup> , smoking	Sudden cardiac death	I/II
12	36/male	HTA <sup>1</sup>	Fatal MI <sup>5</sup>	I/II
13	48/female	None	Lymphoma	I/II
14	72/female	None	Endometrial neoplasia	I/II
15	23/male	Smoking	Myocardiopathy	I/II

Abbreviations: hypertension<sup>1</sup>, systolic BP $\geq$  135; diabetes mellitus<sup>2</sup>; hypercholesterolemia<sup>3</sup> (total-cholesterol > 5,2 mmol/dl); coronary artery disease<sup>4</sup>; myocardial infarction<sup>5</sup>

**Table 2.** Immunostaining with studied markers in individual patients

Subjects	HIF1 alpha	CD30	CRP	IL-6	CD105
1	+++	+	+++	+	+++
2	+++	+	+++	+	+++
3 <sup>1</sup>	+++	0	+	+	+++
4	0	0	+++	+	++
5	+	0	+++	+	++
6	+++	+	+++	+	+++
7	++	0	+++	+	+++
8	++	0	++	+	++
9	+	0	++	+	++
10 <sup>1</sup>	0	0	+	+	0
11	++	0	++	+	+++
12	++	0	+	+	+++
13 <sup>1</sup>	+	0	+	0	+++
14 <sup>1</sup>	0	0	+	+	+++
15	0	0	+	+	+

0, no staining detected; +, weak staining; ++, moderate staining; +++, extensive staining, <sup>1</sup>Patients with no vascular risk factors

(ABC) avidin-biotin peroxidase complex (Vector Laboratories) was applied, and the slides were incubated at RT for 30 minutes. Colour was developed using diaminobenzidine (DAB) and sections were counterstained with haematoxylin before dehydration, clearing, and mounting. Negative controls in which the primary antibody was replaced with PBS were used to test for non-specific binding (data not included). All immunostaining was assessed by 2 investigators simultaneously using a double-headed light microscope. The extent of staining was graded according to a semi-quantitative scale of 0 to + + +: 0, no staining detected; +, weak staining; ++, moderate staining; + + +, extensive staining. Results are presented in table 2.

## 4. RESULTS

Haematoxylin and eosin sections were classified as AHA in type I, initial lesion; II, fatty streaks; III, intermediate lesions; IV, atheroma and V, fibroatheroma (Table 1).

### 4.1 Immunohistochemistry in type IV/V lesions

In type IV/V lesions taken from patients with low to moderate carotid stenosis there was highly selective

staining with HIF-1alpha, CD30, CD105, CRP and IL-6 antibodies in inflammatory areas. Moreover, HIF-1alpha, CRP, CD105 and IL-6 staining was increased in endothelial cells of numerous neovessels. Representative immunostaining taken from patient No. 6 with a type V lesion are presented in Figures 1-5. Figure 6 corresponds to patient No. 9 with a type II-III lesion.

#### 4.1.1. Immunohistochemistry of HIF-1alpha

There was a strong staining in neovessels surrounding lipidic core and in the neovessels of the neointima. Furthermore, inflammatory cells were strongly stained with anti-HIF-1alpha antibody. In the fibrous cap staining was much weaker or absent (Figure 1). Normal looking neovessels (Figure 1a) and collapsed, partially thrombosed neovessels (Figure 1b) were strongly stained.

#### 4.1.2. Immunohistochemistry of CRP

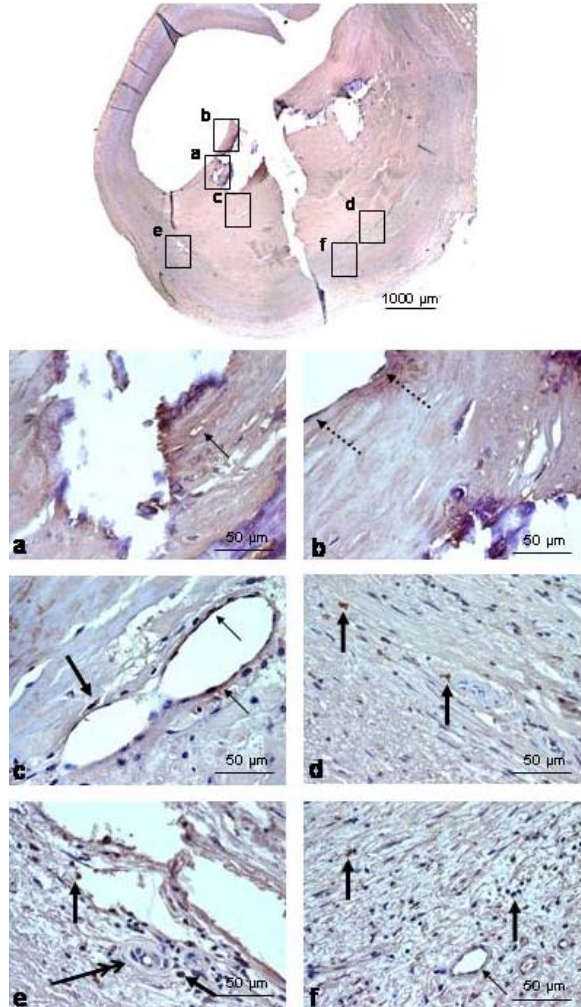
There was a strong staining in the inflammatory cells and within newly formed blood vessels localized in the lipidic core and at the vicinity of thrombus (Figure 2 a-d). CRP (+) areas were mainly present within the fibrous cap (Figure 2 a-c). Moreover, intraluminal endothelial cells were intensively stained (Figure 2b). Interestingly, numerous neovessels in the inflammatory areas were negatively stained (Figure 2e). Areas surrounding the lipidic core rich in inflammatory cells and neovessels were strongly stained (Figure 2f).

#### 4.1.3. Immunohistochemistry of IL-6

There was a strong staining of inflammatory cells and some endothelial cells in the areas of inflammation and angiogenesis. Mainly areas with inflammatory cells close to the highly irregular, partially collapsed vessels were stained (Figure 3a-d). IL-6 staining was visible in the areas surrounding the lipidic core (Figure 3 a, c-d) and in the inflammatory cells and endothelial cells within the lipidic core (Figure 3b). Areas corresponding to plaque shoulders were strongly stained (Figure 3c).

#### 4.1.4. Immunohistochemistry of CD30

Activated B and T-lymphocytes were strongly stained in the areas surrounding lipidic core (Figure 4a) and prone to rupture, plaque shoulders (Figure 4b) at the



**Figure 1.** Immunohistochemistry for HIF-1alpha. Positive HIF-1alpha immunostaining corresponding to neovessels (thin arrows) and inflammatory cells (thick arrows). Normal looking neovessels (insert a) and collapsed, partially thrombosed neovessels (insert b).

vicinity of neovessels. Some of CD30 (+) cells were localized close to the intramural thrombus suggesting its possible implication in the thrombotic events. Furthermore, CD30 (+) cells were present within the non-inflammatory areas (Figure 4 a-b).

#### 4.1.5. Immunohistochemistry of CD105

There was a strong staining in morphologically different neovessels within the carotid plaque. There were highly irregular, multilobular, partially collapsed vessels (Figure 5a, d), flattened and elongated but patent vessels (Figure 5b) and regular-shaped circular vessels (Figure 5c). CD105 positive cells were mainly localized within vulnerable plaque shoulders (Figure 5a, b, d), and within the lipidic core (Figure 5c).

#### 4.2. Immunohistochemistry in type II/III lesions

In patients with type II/III lesions there was a weak staining present in the limited number of inflammatory and angiogenic areas for HIF-1alpha (Figure

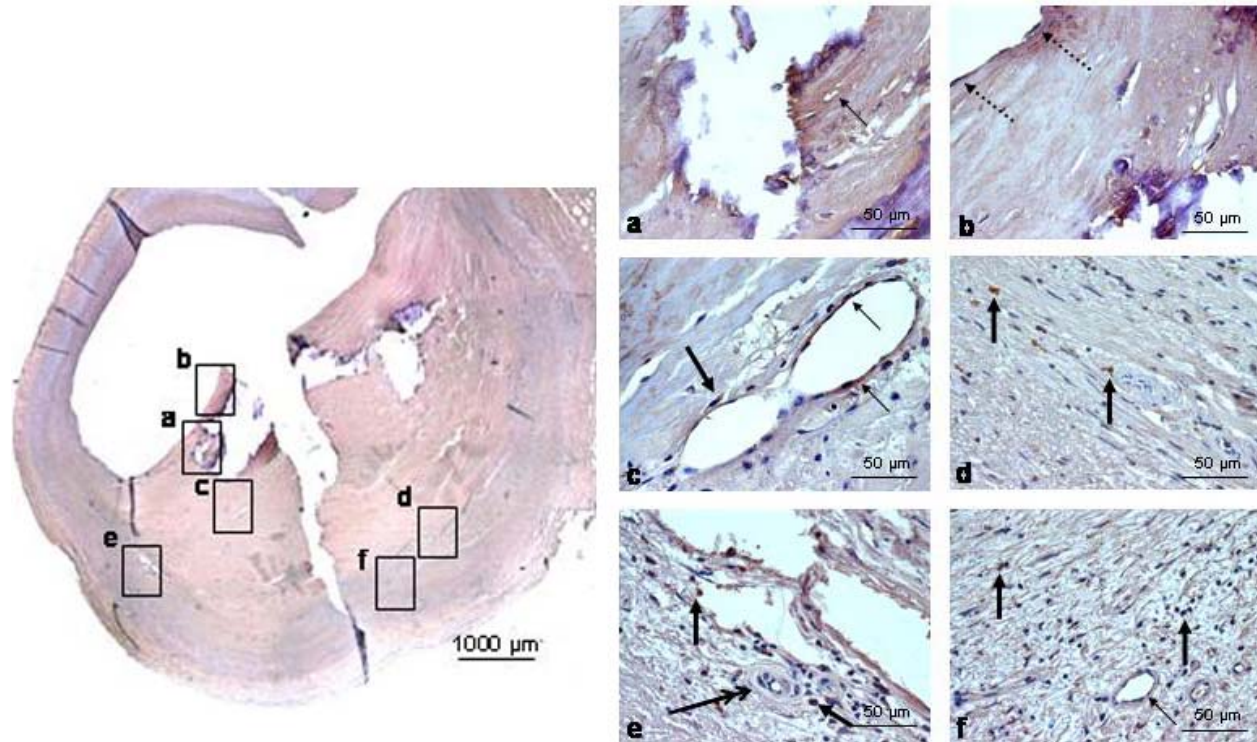
6a), IL-6 (Figure 6b), CRP (Figure 6c) and CD105 (Figure 6d). There was no staining for CD30 in these areas (Figure 6e).

## 5. DISCUSSION

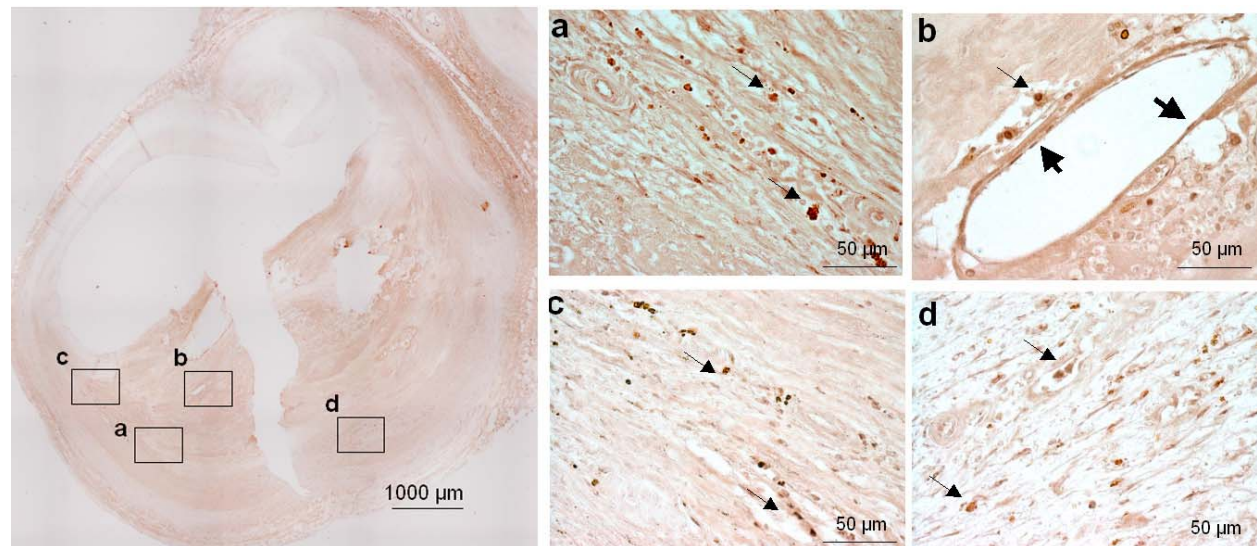
Angiogenesis is implicated in the development and progression of atherosclerosis and is associated with clinical syndromes in the coronary and carotid circulation (14, 26, 27). Little is known about angiogenic responses in the initial to moderate lesions, frequently found in patients suffering from the ischaemic stroke. Angiogenesis is extremely rare in the absence of atherosclerosis, suggesting that intimal disease occurs first and angiogenesis follows. The molecular mechanism responsible for neovessel formation is predominantly linked to hypoxia (28). The latter is a strong HIF-1alpha inducer (7). We observed an important expression of HIF-1alpha in macrophage rich areas of carotid arteries in all types of lesions from type II-V taken from the patients with carotid stenosis under 50%. These results are in accordance with a previous study of HIF-1alpha expression in different plaque phenotypes (7). We also observed abundant expression of HIF-1alpha in hypoxic areas where there was a strong staining in neovessels. This suggests that HIF-1alpha may have a putative role in atherosclerotic progression in patients with low to moderate stenosis. In the contrary, Vink *et al* observed more microvessels in plaques without nuclear HIF-1alpha than in plaques with HIF-1alpha. The authors concluded that a hypoxic environment within plaques with little or no microvessels induces expression of HIF-1alpha. In our series, some patients had no previous cardiovascular risk factors. However, these patients presented with progressing atherosclerosis as observed on histology sections. Furthermore, these lesions had notable expression of studied markers suggesting that there are some other mechanisms involved in atherosclerosis progression beyond the classical risk factors.

An important aspect of newly forming blood vessels is their morphology. Incomplete blood vessels, without basal lamina are weak and prone to leakage leading to intraplaque haemorrhage. The latter is often observed in symptomatic carotid plaques (29). McCarthy *et al* described the large variation in the shape of the neovessels. They divided neovessels shape into three groups: (a) highly irregular, multilobular, partially collapsed vessels, (b) circular regular-shaped vessels, and (c) flattened and elongated but patent vessels (30). They suggested that in addition to plaque microvessel density, it is possible that the phenotype of intraplaque vessels may influence plaque stability (31). We observed that microvessels were most abundant in type IV/V plaques. There was a clear over-expression of CD105 in these atheromatous lesions. CD105 co-existed with HIF-1alpha in neovessels surrounding the lipidic core and in the neointima. These vessels shape were type (a) and (c). We suggest that these type of vessels are more prone to leak and break, and increase the possibility of progression to unstable lesion. Further, CD105 was also expressed in neovessels localized in the lipidic core, where vessels were predominantly circular and of regular-shape.



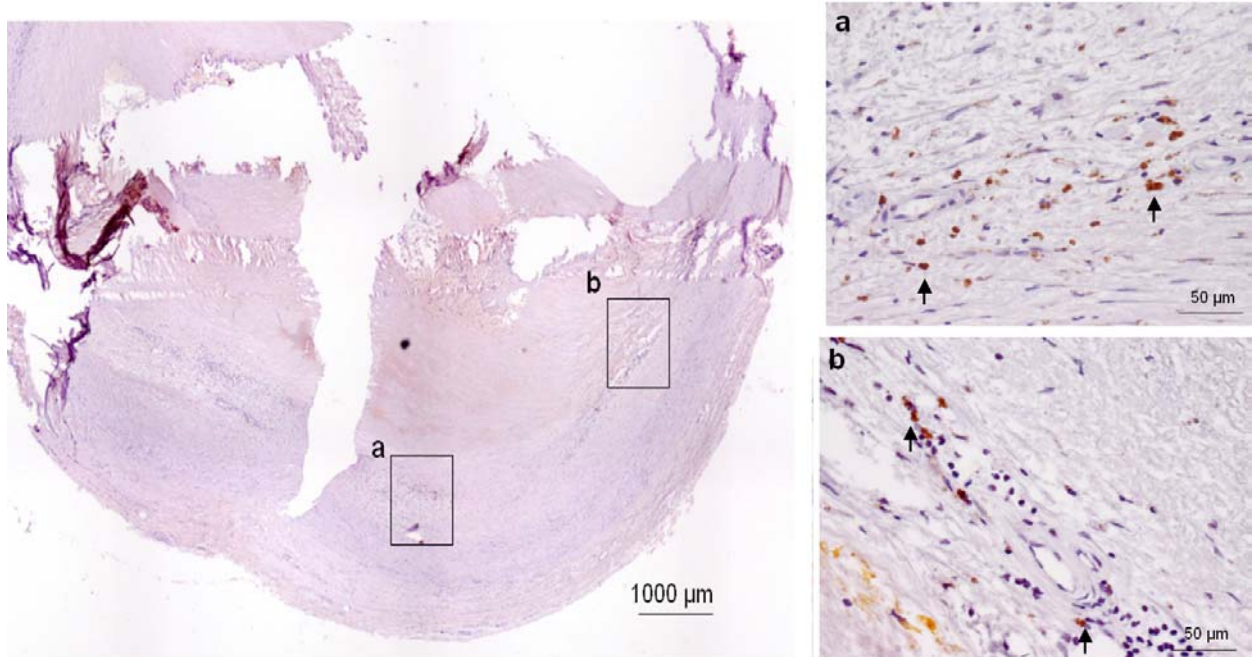


**Figure 2.** Immunohistochemistry for CRP. Positive CRP immunostaining corresponding to neovessels (thin arrows), inflammatory cells (thick arrows) and endothelial cells (break arrows). Negative CRP immunostaining in neovessels of the inflammatory areas (double arrows). Endothelial cells corresponding to the neovessels within the thin fibrous cap (insert a) and lumen lining endothelial cells were strongly stained (insert b). There was staining in the patent neovessels within the lipidic core (insert c). Numerous inflammatory cells were stained (inserts d-f). However, some neovessels were negative for CRP (insert e). Areas surrounding lipidic core were strongly stained (insert f).

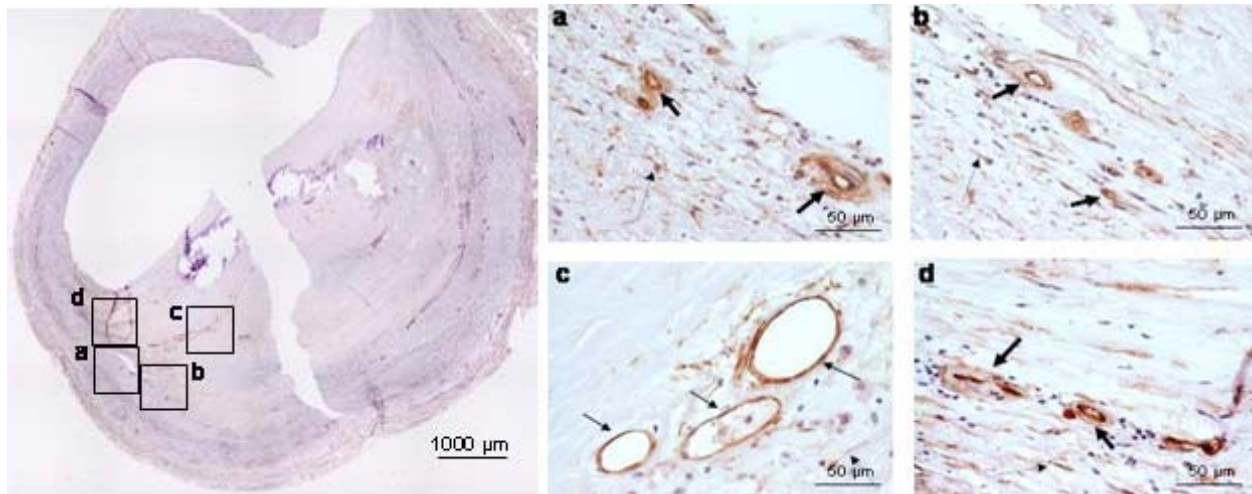


**Figure 3.** Immunohistochemistry of IL-6. Positive IL-6 immunostaining corresponding to inflammatory cells (thin arrows) and endothelial cells (thick arrows). IL-6 staining was visible in the areas surrounding the lipidic core (inserts a, c, d) and in the inflammatory cells and endothelial cells within the lipidic core (insert b). Areas corresponding to so called plaque shoulders were strongly stained (insert c)

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**Figure 4.** Immunohistochemistry CD30. Positive CD30 immunostaining corresponding to activated B- and T-lymphocytes. B- and T-lymphocyte rich-areas surrounded the lipidic core (insert a) and were localized in the plaque shoulders (insert b).



**Figure 5.** Immunohistochemistry of CD105. Positive CD105 immunostaining corresponding to different phenotype neovessels: highly irregular, multilobular, partially collapsed vessels (inserts a,d), flattened and elongated but patent vessels (insert b) and regular-shaped circular vessels ( insert c).

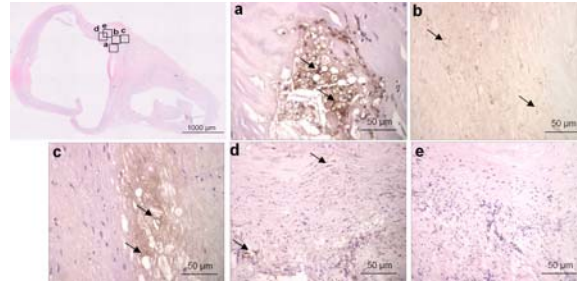
We observed abundant expression of CRP in type IV/V lesions but not in type II/III lesions. In studied atheromas there was increased number of neovessels and infiltrating cells, including macrophages and activated B- and T-lymphocytes, predominantly in hypoxic areas. CRP was highly expressed in these cells. Interestingly, numerous neovessels did not express CRP within the inflammatory areas. CRP was mainly observed in the areas of thin fibrous cap and at the vicinity of intraplaque thrombi. This observation suggests that CRP is involved in modulation of

angiogenesis and maybe be involved in acute plaque events (1, 9). It is possible that CRP by inhibiting angiogenesis may stabilize atheroma (32).

It is likely that a local immune responses occur in the atherothrombotic plaque, but its pathophysiologic consequences remain largely speculative. Cellular immune responses may initiate inflammatory reactions, cell-mediated cytotoxicity, and cytokine-dependent regulatory loops in the atherothrombotic plaque (33). We observed that activated B- and T-lymphocytes were



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**Figure 6.** Representative type II/III lesion in patient No. 9. Immunohistochemistry of HIF-1alpha (a). Immunohistochemistry of CRP (b). Immunohistochemistry of IL-6 (c). Immunohistochemistry of CD105 (d). Immunohistochemistry CD30 (e).

present within non-inflammatory areas and possibly participate in the response which is chemotactic for the inflammatory cells.

The localization and increased expression of angiogenic growth factors, as well as HIF-1alpha, within the plaque and the localization of macrophages and activated B and T-lymphocytes adjacent to these vessels may result in excess remodelling of nearby vessels and further contribute to plaque progression to unstable lesion. Our findings support the hypothesis that dynamic changes in the non-occluding plaques may indeed explain some of the clinical events in patients with low to moderate carotid stenosis.

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**Abbreviations:** HIF-1alpha: hypoxia-inducible factor-1alpha, IL-6: interleukin-6, CRP: C-reactive protein, bHLH-PAS: basic helix-loop-helix-PAS, VSMC: vascular smooth muscle cells, TGF-beta1: transforming growth factor beta 1, TNF: tumor necrosis factor, AHA: american heart association, ABC: avidin-biotin peroxidase complex, DAB: diaminobenzidine.

**Key Words:** Hypoxia, Inflammation, Carotid Atherosclerosis

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