Abstract. There is a strong correlation between macrophage infiltration and plaque instability in recently symptomatic carotid atherosclerotic plaques, and it is hypothesised that mechanisms related to macrophages may be involved in plaque vulnerability and rupture. We previously found high expression of urokinase-type plasminogen activator receptor (UPAR) in human macrophages. The aim of this study was to investigate whether UPAR co-localises with macrophages in symptomatic carotid plaques, and whether UPAR expression is associated with plaque rupture. Real-time RT-PCR assays showed that UPAR expression levels were high in monocyte-derived macrophages and in carotid endarterectomies compared with a tissue panel. Serial transverse sections were prepared from carotid endarterectomies from 12 symptomatic patients, and analyzed with immunohistochemical staining for UPAR and for CD68-positive macrophages, and with histopathological assessment. UPAR co-localised with CD68-positive macrophages, with a high correlation (r=0.90, p<0.001) between immunostained areas in 12 carotid endarterectomies from symptomatic patients. High degrees of UPAR and CD68 staining were found in sections around the bifurcation level where rupture was most common, while low degrees of staining were found in sections of the common carotid artery end of the endarterectomy (p<0.05). Higher degrees of UPAR staining were observed in ruptured plaque sections compared with non-ruptured sections. In conclusion, UPAR was highly expressed in monocyte-derived macrophages and in symptomatic carotid plaques. UPAR co-localised with macrophages in carotid symptomatic plaques and UPAR was predominantly found in ruptured plaque segments. These findings support the hypothesis that UPAR is related to plaque rupture in symptomatic atherosclerotic lesions.

Introduction

It is well known that some, but not all, highly stenotic carotid atherosclerotic lesions confer increased risk of clinical diseases such as transient ischemic attacks and strokes (1,2). Such symptomatic carotid lesions are usually associated with complicated ruptured atherosclerotic plaques (2,3). It is still not clear why some plaques are vulnerable with an increased risk of rupture and complications such as thrombosis. Accumulating data show that there is a strong correlation between macrophage infiltration and plaque vulnerability in recently symptomatic carotid plaques (2-4). Thus, we postulated that genes that are highly expressed in monocyte-derived macrophages could be of importance in plaque vulnerability (5).

In a DNA microarray-based screening study, we recently observed that expression of the urokinase-type plasminogen activator receptor (UPAR) was more than 9-fold higher in monocyte-derived macrophages compared with a panel of 55 other human tissues and cell types (5). UPAR is a glycoprotein (55-60 kDa) that has been associated with cell adhesion, tissue remodelling, and neo-vascularisation (6,7). It is expressed on the surface of many cell types, including macrophages and neoplastic cells (5,8,9), and its expression level correlates with the migratory and invasive potential in different tumor diseases (10). Colon, prostate, lung and ovarian cancers are associated with an increased UPAR expression (11). Higher protein levels of UPAR have also been found in atherosclerotic
lesions compared with normal vessels (12). In addition, a study in human coronary arteries from explanted hearts and aortic vessels obtained by autopsy has shown that the UPAR content is higher in fibrous plaques compared with early lesions or normal arterial tissue (13). However, it is not known whether plaque rupture leading to acute clinical diseases such as strokes or transient ischemic attacks is associated with high UPAR expression.

The aim of this study was to investigate whether UPAR co-localises with macrophages in symptomatic carotid plaques, and whether UPAR expression is associated with plaque rupture in patients with symptomatic atherosclerotic lesions.

Materials and methods

Study sample. Göteborg Atheroma Study Group (GASG) is a biobank consisting of clinical information, blood, plasma, serum and endarterectomies from white European patients with symptomatic carotid stenosis. Here, two sub-groups of patients from this biobank were analyzed.

Subgroup 1: Frozen endarterectomies from 6 randomly selected patients were used for gene expression studies. Median age was 69.5 (range 60-82) years, 6/6 were men, 4/6 had hypertension, 0/6 had diabetes, 2/6 had suffered a myocardial infarction, and 4/6 were on statin treatment.

Subgroup 2: Paraffin-embedded endarterectomies from 12 patients were used for immunohistochemical and histopathological studies. Median age was 65 (range 56-74) years, 6/12 were men, 10/12 had hypertension, 4/12 had diabetes, 1/12 had suffered a myocardial infarction, and 10/12 were on statin treatment.

Approval was obtained from the Regional Ethics Review Board at Göteborg University, and all subjects gave informed consent to participate.

UPAR gene expression in cultured macrophages and endarterectomies. Cultured macrophage RNA from 4 anonymous blood donors was isolated as previously described (14). Plaque RNA was obtained from 6 carotid endarterectomies (subgroup 1) using the method of Chomczynski and Sacchi (15). cDNA was generated using the High Capacity cDNA Archive Kit from Applied Biosystems. Gene expression of UPAR in cultured macrophages and carotid plaques was compared with expression in various tissues of a reference tissue panel, constructed using the Human Total RNA Master Panel II (K008-1) obtained from Clontech (Mountain View, CA).

Immunohistochemical and morphological analyses of endarterectomies. Carotid endarterectomies from 12 patients (subgroup 2) were fixed in formalin immediately after surgical removal, divided transversely into sections ~3-mm thick, and embedded in paraffin blocks. Serial transverse 4-μm sections were taken from each block for morphological analysis and immunohistochemistry. In total, 127 sections were analyzed. The sections were de-waxed and treated with antigen retrieval reagent (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

To identify macrophages and smooth muscle cells, sections were treated with a CD68 mouse monoclonal antibody (Novocastra Laboratories, Newcastle, UK; 1:500 dilution) or an anti-muscle α-actin mouse monoclonal antibody (Enzo Diagnostics, Farmingdale, NY; 1:1500 dilution), together with a universal HRP-DAB staining kit (Dako, Glostrup, Denmark).

To identify UPAR, sections were treated with an anti-human UPAR goat polyclonal antibody (R&D Systems; 1:200 dilution) together with an anti-goat HRP-DAB staining kit (R&D Systems). Blocking and staining were carried out according to the manufacturer's instructions, modified by supplementing PBS buffer for UPAR staining with 0.1% saponin. Incubation with a primary antibody was performed overnight in a humidity chamber at 20°C. UPAR sections were counter-stained with haematoxylin. As a negative control, PBS buffer was substituted for the primary antibody. Human tonsil tissue was used as a positive control for CD68.

After standardized microscopy and digitalization with a Zeiss Axioplan 2 imaging microscope and using a Zeiss Axioscam digital camera (Göttingen, Germany), the stained areas were quantitated using the BioPix Tissue 1.5 software (BioPix AB, Gothenburg, Sweden; http://www.biopix.se) in accordance with the manufacturer's instructions. The Bio-Pix software was previously characterized for automatic quantitative analysis of stained biological samples (17). Stained areas were automatically identified by the color and intensity spectra using the same thresholds for all images. Software-identified areas were followed interactively by semi-transparent overlays to assure that all labeled material was found, without detecting potential artefacts. The total sectioned area was identified by combining manual and automatic methods. CD68- and UPAR-stained areas were identified using three different threshold settings that showed statistically significant correlations, and only data from one threshold setting are thus presented (hue, 0.0-0.5; saturation: 0.12-1.0; brightness, 0.0-1.0).

To test the reproducibility, 9 sections were analyzed by 2 investigators on two occasions, at least 3 days apart. Intra-observer coefficients of variation for the 2 investigators were 0.50 and 0.66%, and the interobserver coefficient of variation was 1.02%.

One section from each 3-mm block from the 12 endarterectomies was stained with Mayer's haematoxylin, and 1 investigator classified each section according to the AHA classification (18). To test the reproducibility of the classification, 89 sections from 30 carotid plaques outside this study were analyzed by the same investigator on two occasions with an interval of 2 weeks, showing a good reproducibility ($r_{p}=0.82, p<0.001$).

UPAR expression was compared between sections with and without plaque rupture. For each endarterectomy, the median relative immunostained UPAR area was determined for sections classified as AHA VI (ruptured sections). This was repeated for the remaining, non-ruptured sections (ranging from AHA III to V), and the difference in median relative immunostained area between the groups was calculated. One endarterectomy (patient 8) did not contain any sections classified as AHA VI, and was excluded from this analysis.
Statistical analysis. Results are represented as median and inter-quartile range (IQR), or as a percentage for categorical variables. 95% confidence intervals (CI), Wilcoxon’s non-parametric test, Fisher’s exact test, and Spearman’s correlation co-efficient ($r_s$) were used in the statistical analyses. $p<0.05$ (two-sided) was regarded as statistically significant.

Results

High gene expression of UPAR in cultured macrophages and endarterectomies. Gene expression levels of UPAR were higher in cultured macrophages (UPAR/PPIA mRNA expression: mean 1.48, 95% CI 1.14-1.82) and carotid endarterectomies (mean 1.59, 95% CI 1.58-1.59) compared with tissues from the reference panel (mean 0.26, 95% CI 0.13-0.38). UPAR expression was 1.4-fold higher in macrophages and 1.5-fold higher in carotid endarterectomies than in bone marrow - the reference tissue with the highest level of expression (Fig. 1).

High correlation between UPAR and CD68 in endarterectomies. In paired transverse sections of carotid endarterectomies, we observed that UPAR staining co-localised with staining for CD68 (a marker of macrophages) but not with $\alpha$-actin (a marker of smooth muscle cells) (Fig. 2A-C). Human tonsillar tissue stained positive for CD68, whereas no staining was noted for UPAR (Fig. 2D and E).

In 11 of the 12 samples, analyses of all available paired tissue sections showed a close correlation between UPAR and CD68-positive macrophages (Table I). Comparison of the paired median values from each of the 12 endarterectomies showed a statistically significant correlation between UPAR- and CD68-stained areas (Fig. 3) but no correlation between UPAR- and $\alpha$-actin-stained areas ($r_s=0.06$, data not shown).

Association between degree of plaque instability and UPAR- and CD68-stained areas. We assessed the distribution of UPAR and CD68 staining longitudinally across the endarterectomies at 3-mm intervals, and related this to the histopathological classification. There were significant differences in the degree of staining, and in the frequency of rupture (AHA VI), between different segments of the plaques. Immunostained UPAR and CD68 areas were larger in sections around the bifurcation than in sections of the common carotid artery end of the endarterectomy (Fig. 4A and B). Similarly, plaque rupture (AHA VI) was more frequent around the bifurcation when compared with the common carotid artery end of the endarterectomy (Fig. 4C).

Furthermore, higher degrees of UPAR staining were found in AHA VI-classified sections of the endarterectomies than in AHA III-V-classified sections (Table II). Hence, relative UPAR-stained areas were largest in complicated ruptured segments of the plaques.

Discussion

In this study, we showed high expression of UPAR in human macrophages and in carotid endarterectomies from patients with symptomatic atherosclerosis, we demonstrated a close co-localisation of UPAR and CD68-positive macrophage staining in human endarterectomies, and observed the highest degrees of UPAR staining in ruptured sections within the endarterectomies.

Previous studies have described an increased UPAR expression in atheroma by immunohistochemistry or Western blot analysis (12,19). Steins et al measured UPAR content in...
tissue extracts from coronary arteries from explanted hearts and aortas obtained by autopsy, and showed that the UPAR content increased progressively with the severity of atherosclerosis (13). However, they examined only asymptomatic fibrous and calcified plaques and not symptomatic ruptured plaques, and did not investigate the longitudinal distribution of UPAR within plaques. Our results support and extend these previous studies as they demonstrate that UPAR was unevenly distributed within carotid symptomatic plaques, with higher degrees of UPAR staining in ruptured segments.

Table II. UPAR immunostained areas in ruptured (AHA VI) and non-ruptured (AHA III-V) sections of endarterectomies from symptomatic patients with carotid stenosis (n=11).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>AHA VI-classified sectionsb</th>
<th>AHA III to V-classified sectionsb</th>
<th>Difference in UPAR stained areas between sections with AHA VI and AHA III-V (percentage points) c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>UPAR-stained area (% of section)</td>
<td>n</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>5.30</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1.74</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.17</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>3.98</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
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<td>4.83</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
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<td>8</td>
</tr>
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</tr>
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</tr>
<tr>
<td>12</td>
<td>1</td>
<td>2.36</td>
<td>6</td>
</tr>
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</table>

Average (95% confidence interval of differences) 2.7 (1.5-3.8)

aThe endarterectomy from patient 8 did not contain any sections classified as AHA VI. bEach section was classified according to the AHA criteria of plaque vulnerability (18). cMedian relative UPAR-immunostained area of all AHA VI-classified (ruptured) sections of the individual endarterectomy. dMedian relative UPAR-immunostained area of all AHA III to V-classified sections of the individual endarterectomy. eDifference in median relative UPAR-immunostained area between AHA VI- and AHA III to V-classified sections of the individual endarterectomy.
of the plaques. We also demonstrated a highly significant association between UPAR and macrophages within carotid symptomatic plaques.

The uneven distribution of UPAR in the plaques has important implications from a methodological perspective. When sections are taken from symptomatic carotid plaques for studies of UPAR and UPAR-related mechanisms, it is important to realise that UPAR expression differs within the plaques. The highest expression is, in general, found around the bifurcation level, whereas it is lowest in the common carotid artery end of the endarterectomies. Comparisons of sections taken from different locations of the plaques should be performed with care.

We did not observe UPAR staining in human tonsillar tissue in which macrophages are abundant, suggesting that UPAR synthesis is not identical in macrophages from different sites in the body. It has been shown that cultured human monocytes release UPAR when stimulated by oxidized low-density lipoprotein (20). Thus, it is possible that synthesis of UPAR in plaque macrophages is stimulated by oxidized low-density lipoproteins in atherosclerotic lesions. Alternatively, UPAR gene expression could be induced by hypoxia which is a prominent feature, not only in atherosclerotic plaques, but also in malignant tumours, rheumatoid arthritis, and healing wounds (21). Macrophages respond rapidly to hypoxia by altering their expression of a wide array of genes (21). A role of UPAR is suggested in tumours as well as rheumatoid arthritis (22) and wound healing (23).

There was no association between UPAR and α-actin staining in the endarterectomies, indicating that UPAR is not associated with smooth muscle cells in the plaques. This is in contrast with previous studies that have shown associations between UPAR and periluminal (19) and neointimal smooth muscle cells (10,12). Our results are supported by quantitative analysis with formal statistical testing, but the differences in the results may also be explained by other methodological issues.

This study followed our attempt to develop a search strategy for finding genes associated with plaque rupture (5). Using DNA microarrays, we had previously identified UPAR as a gene predominantly expressed in monocyte-derived macrophages compared with other tissues (5). Here, we verified this finding in macrophages using real-time RT-PCR, and in addition we demonstrated high expression levels in carotid endarterectomies from symptomatic patients. Using immunohistochemistry, we then demonstrated the co-localisation of UPAR and macrophages in carotid endarterectomies, and the association between UPAR and plaque rupture. This multi-step approach to identify candidate genes for plaque rupture...
proved successful, and can be applied to identify other candidate genes.

The study was limited by including only symptomatic plaques, and thus it can not be concluded as to whether the increased UPAR expression was causative or secondary to the plaque rupture. A recently published study has shown that plasma levels of soluble UPAR were associated with carotid plaques in haemodialysis patients, supporting the concept that UPAR is involved in plaque development (24).

In conclusion, we demonstrated here that UPAR is expressed in symptomatic atherosclerotic plaques, and that UPAR associates with CD68-positive macrophages and with ruptured plaque segments in carotid endarterectomies from patients with symptomatic atherosclerosis. These results support the hypothesis that UPAR is related to plaque rupture in symptomatic atherosclerotic plaques.

Acknowledgements

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References


