Novel visualization techniques towards identification of atherosclerotic patients at risk

Jager, Nynke

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CHAPTER 6

Targeted folate receptor β fluorescence imaging as a measure of inflammation to estimate vulnerability within the human atherosclerotic carotid plaque

Nynke A. Jager, Johanna Westra, Gooitzen M. van Dam, Nato Teteloshvili, René A. Tio, Jan-Cees Breek, Riemer H.J.A. Slart, Hendrikus Boersma, Phillip S. Low, Marc Bijl, Clark J. Zeebregts

Abstract

OBJECTIVES The probability of atherosclerotic plaque rupture and its thrombotic sequelae are thought to be increased at sites of macrophage accumulation. Folate receptor β (FR-β) is present on activated macrophages but not on quiescent macrophages or other immune cells. By conjugating the ligand folate with a fluorescent contrast agent, fluoresceine-isothiocynate (FITC), we aimed to explore the potential role of folate receptor β fluorescence imaging in the distinction of vulnerable sites from more stable regions.

METHODS Carotid specimen were taken from a total of 20 patients and incubated with folate-FITC for 30 minutes. Ex vivo fluorescence imaging with an IVIS Spectrum® was performed to determine the exact location of folate-FITC uptake. Sections displaying regions of high uptake (determined as hot spots) were compared with sections showing low uptake (cold spots) through immunohistochemistry and real time quantitative reverse transcription PCR for FR-β.

RESULTS Hot spots showed significantly higher folate-FITC uptake compared to cold spots (p<0.001). Hot spots tended to contain more macrophages and areas of hypoxia compared to cold spots. A positive correlation between mRNA levels of CD68 (marker for macrophages), folate receptor β (r=0.53, p=0.045) and HIF-1α expression (marker for intraplaque hypoxia; r=0.55, p=0.034) was found.

CONCLUSION In areas of high folate-FITC uptake within human atherosclerotic plaques, an increased number of activated macrophages and higher areas of hypoxia were present compared to areas with low folate-FITC uptake. These characteristics of vulnerability imply that molecular imaging of folate receptor-β through folate conjugates might be a good indicator for plaque vulnerability in future non-invasive imaging studies.

Keywords: Carotid artery; atherosclerotic plaque; vulnerability; folate receptor-β; fluorescence imaging.
FOLATE-FITC IMAGING; MAKING MACROPHAGES VISIBLE IN PLAQUES

Introduction

Atherosclerosis can be regarded as a progressive inflammatory process of the arterial wall ultimately leading to the formation of plaques. Currently, cardiovascular atherosclerotic disease is the leading cause of death in men and women in the western world (1). Atherosclerotic thickening of the vascular wall may compromise end-organ blood flow, but the greatest risk for clinical events is present in patients with an unstable plaque. Rupture of such a vulnerable plaque is one of the predominant underlying processes in the pathogenesis of both myocardial infarction and ischemic stroke. Histological assessment of ruptured plaques has shown several distinctive features, including a large lipid core composed of free cholesterol, oxidized lipids, a thin fibrous cap depleted of smooth muscle cells and collagen, inflammatory cell infiltration and increased adventitial and plaque neovascularization (2). Currently, indications for interventional treatment are based on clinical symptomatology and degree of stenosis (>70%) (3, 4). However, it has been recognized that plaque vulnerability rather than plaque size or severity of stenosis is important for plaque rupture risk assessment (5). In this context, the extent and location of plaque inflammation appear to be key factors in determining plaque vulnerability (6). In current clinical practice, anatomic imaging modalities, such as B-mode duplex ultrasound, computed tomography angiography scanning, and magnetic resonance imaging, can identify stenotic plaques of the internal carotid artery, but they give little or no information regarding molecular and cellular processes (5, 6).

Previous studies have shown that inflammation is related to the accumulation of activated macrophages at sites of plaque rupture (7). For this reason, the activated macrophage has emerged as an important diagnostic and therapeutic target for atherosclerosis (8, 9). The folate receptor β (FR-β) is present on activated macrophages but not on quiescent macrophages or other immune cells (10-12). Another family member, folate receptor α, is expressed on many epithelial cancers (13), while folate receptor γ is secreted by hematopoietic cells at levels generally too low to detect by standard methods (14). Folate receptor δ is restricted to regulatory T cells and for unknown reasons does not bind folate (15). In general, the folate receptor (FR) is a 38 kDA glycosylphosphatidylinositol-anchored protein that binds to the vitamin folic acid with high affinity (KD<1nM) (12).

By visualizing the FR-β with an optical fluorescent contrast agent, activated macrophages can be detected by optical imaging. As such, van Dam et al. conjugated fluoresceine-isothiocyanate (FITC) with folate (i.e. folate-FITC) to identify tumor processes, as several solid tumors express folate receptor α (16). Similarly, Ayala-López et al. showed a folate-99mTc-EC20 compound binds to the FR-β and therefore localizes folate in apolipoprotein E knock-out mice fed a western diet (11).

Vulnerable plaques are also characterized by intra-plaque hypoxia (17). The transcription factor hypoxia-inducible factor-1 alpha (HIF-1α) is regarded as the major oxygen homeostasis regulator. During hypoxia, HIF-1α levels rapidly increase and dimerize to HIF-
1β to form the stable heterodimer transcription factor HIF-1. HIF-1 plays an important role in angiogenesis and is involved in tumor growth leading to resistance to radiotherapy and chemotherapy, as well as in inflammatory reactions (18, 19).

The aim of this pilot study was to explore the potential of fluorescence labeled folate as a macrophage activity marker in vessel wall. Thus we investigate whether areas of high folate-FITC uptake, reflect the presence of an increased number of activated macrophages, characteristic of an unstable plaque in the internal carotid artery. In addition, we investigated HIF-1α expression in atherosclerotic plaques, and compared this to the distribution of FR-β within the plaque in order to confirm the relationship between hypoxia and plaque vulnerability. These data will contribute to the development of optical imaging of the carotid artery in the near future that could be used as a non-invasive imaging method to detect and quantify the vulnerability of atherosclerotic plaques.

Materials and methods

Study Design
Between October 2008 and April 2009, carotid specimens were taken from a total of 20 patients. These patients underwent open carotid surgery in two hospitals (University Medical Center Groningen (UMCG), and Martini Hospital, Groningen, The Netherlands). From these patients atherosclerotic plaques were obtained by means of a carotid endarterectomy (CEA) of the internal carotid artery using standard techniques. Patients who underwent surgical treatment were symptomatic patients (i.e. with a history of cerebrovascular accident (CVA), transient ischaemic attack (TIA) or amourosis fugax) who had a stenosis of 70-99% as detected by duplex ultrasound. Also asymptomatic patients with a stenosis of 80-99% were eligible for surgical treatment (UMCG only). Risk factors such as smoking status, obesity (body mass index, BMI), hyperlipidemia, hypertension and diabetes mellitus were recorded. Hypertension was defined as systolic arterial pressure above 140 mmHg and/or diastolic arterial pressure above 90 mmHg, or use of antihypertensive drugs, prescribed with the aim to reduce blood pressure (20). Hyperlipidemia was diagnosed if plasma total cholesterol exceeded 6.21 mmol/l, plasma LDL cholesterol exceeded 3.36 mmol/l, plasma triglycerides exceeded 2.26 mmol/l, or if the patient used lipid-lowering drugs (20). The study was approved by the Institutional Review Board (IRB) of each hospital and informed consent was obtained from all patients.

Carotid Endarterectomy Sample Collection And Timepath Of Study
The carotid samples were collected during CEA. The samples were immediately transported in a phosphate buffer on ice to the IVIS spectrum, with a transport time of 21±5 minutes at the UMCG hospital, and 50±14 minutes at the Martini hospital. After the samples were collected and transported, imaging with the IVIS spectrum was performed after determining the optimal incubation time and concentration (figure 2B). To validate
the folate-FITC signal, protein levels (immunohistochemistry, immunofluorescence) and gene-levels (RT-PCR) were measured.

**Imaging Of The Plaque**

*Ex vivo* fluorescence imaging (FLI) was performed to determine the exact location where the folate-FITC (MW 916 kDa, Supplemental figure 1) uptake had taken place within the plaque. Before imaging the concentration and incubation time were determined by making a serial dilution. Several concentrations were tested. Due to the highest intensity found at an incubation time of 30 minutes, in 2 ml of folate-FITC in PBS and a folate-FITC:PBS dilution of 1:10 (10 μg/ml), we considered this as the ideal concentration and incubation time. Fluorescence images were taken with a commercial imaging system with an ultra-sensitive charge-coupled device (CCD) camera mounted on a light-tight black chamber IVIS® Spectrum (Caliper Life Sciences, Hopkinton MA, U.S.A.). In this imaging system we used wavelengths of 500 nm excitation wave, and 540 nm emission wave for every sample. To suppress autofluorescence from surrounding tissue spectral unmixing algorithms were used. The technique of fluorescence imaging with the system has been described previously (22). Sections were taken at locations with high intensity (i.e. > 5.00e-4 radiance, photons/sec/cm²/sr) folate-FITC uptake (so-called hot spots), medium spots (medium uptake, intensity 2.00e-4 till 5.00e-4 radiance, photons/sec/cm²/sr) and cold spots (low uptake intensity < 2.00e-4 radiance, photons/sec/cm²/sr). Cold spots served as negative control tissue. Images acquired were processed using Living Image version 3.0® software (Caliper Life Sciences, Hopkinton MA, U.S.A.).

**Validation Of Folate-FITC Labeling**

To investigate whether FR-β is present on activated macrophages and whether folate-FITC (kindly provided by prof. P.S. Low) binds to FR-β, we repeated the experiment as described by Xia et al.(10). Macrophages expressed in synovial fluid of rheumatoid arthritis patients are considered to be activated because proinflammatory or regulatory cytokines and growth factors, tumor necrosis factor (TNF)-α, granulocyte–macrophage colony-stimulating factor (GM-CSF), chemokines and chemoattractants, metalloproteinases and neopterin are all overexpressed (21). Synovial fluid was obtained from patients with rheumatoid arthritis who had given informed consent, and mononuclear fraction was collected by Ficoll gradient separation. Cells were stained with anti-CD11b-PE (BD Biosciences, California, USA) to distinguish macrophages from other immune cells. To demonstrate presence of FR-β on the macrophages, the receptor was visualized using biotinylated anti-human FR-β antibody (R&D systems). Streptavidine-APC (Biolegend) was used as secondary antibody. These cells were also incubated with folate-FITC (100 nM, 0.05 μg/ml) and to test if this was specific binding we also incubated with 100-fold excess of free folic acid (nr. 1.03984, Merck, Darmstadt, Germany).
CHAPTER 6

Immunohistochemistry
Biopsies were taken of the first 10 patients through the plaque at the sites of ‘hot’, ‘medium’ and ‘cold’ spots represented on the IVIS® spectrum. Subsequently these spots were checked with this system, by making a new fluorescence image to make sure this piece was a hot, medium or cold spot indeed. Next they were embedded in paraffin, and sections of 4 μm were made of all spots of 10 patients. After deparaffinization endogenous peroxidase activity was blocked by 0.3% H₂O₂ incubation for 30 minutes. Macrophages were identified using monoclonal mouse anti-human antibody CD68 antibody (1:50; clone PG-M1 DAKO, Glostrup, Denmark) for 60 minutes at room temperature. Endothelial cells were made visible by using mouse anti-human anti-CD31 monoclonal antibody (1:25) clone JC70A (DAKO) for 60 minutes at room temperature. To visualize areas of decreased oxygen level i.e. hypoxia HIF-1α (hypoxia-inducible factor -1α) stainings were performed with mouse antibody HIF-1α 67sup (Abcam, Cambridge, UK) overnight at 4ºC (23). Labeled polymer-HRP anti mouse from the Dako K4006 kit were used as second antibody. Color reaction was developed using DAB staining with chromogen, and sections were counterstained with hematoxylin. Nuclei were therefore stained blue, and specific immunostaining was shown brown. Immunoreactivity in the plaques was scored by two independent observers as follows: (0 = none; 1 = mild; 2 = moderate; 3 = extensive) (24)

Immunofluorescence
To demonstrate co-localization of folate receptor β with macrophages, fluorescence microscopy with CD68 and FR-β was performed on hot spots and cold spots of atherosclerotic carotid plaque, determined with the IVIS® spectrum. After deparaffinization, antigen retrieval with 10mM tris-HCL + 1 mM EDTA was performed for 60 minutes. Subsequently endogenous peroxidase activity was blocked by 0.3% H₂O₂ incubation for 30 minutes. Macrophages were identified using the same antibody, concentration and incubation time as used with immunohistochemistry. FR-β was visualized using biotinylated anti-human FR-β antibody (1:10) overnight at 4ºC (R&D systems). TRITC-goat anti-mouse IgG3 (Southern Biotech) and streptavidine-APC (Biolegend) were used as secondary antibodies respectively. DAPI staining was performed to stain nuclei. Pictures were taken using the Quantimet (Leica, 600S).

RNA Isolation, cDNA Synthesis And qRT-PCR
In this study, it appeared that RNA-isolation from the imaged plaques was not possible due to breakdown of RNA during folate-FITC incubation at room temperature. During the process of incubation for 30 minutes, RNA remained stable (and detectable) with rtPCR for only a few minutes. To investigate mRNA expression of relevant proteins in atherosclerotic plaques we used biopsies from atherosclerotic plaques from the carotid artery directly stored at -80 ºC immediately after explantation, which had not been imaged in the IVIS Spectrum. RNA isolation, cDNA synthesis and qRT-PCR were performed. In short, TRIzol was added to
the frozen tissues, which were then ground with a pestle. Total RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). The reverse transcriptase step was performed using reagents from Invitrogen (Breda, the Netherlands). One μg of total RNA was transcribed to cDNA with 0.5 μl 7.5 μM Oligo (dT) 24 primers, 1 μl M-MLV Reverse Transcriptase (RT) enzyme, 5 x first strand buffer, 0.9 μl 0.1 mM DTT, 25 mM dNTP’s in a total volume of 25 μl RNAse free water. For measurement of mRNA 1 μl cDNA sample was added in duplicate for amplification by the Taqman real time PCR system (ABI Prism 7900HT Sequence Detection system, Applied Biosystems, Foster City, CA, USA). Investigated genes were: GAPDH, CD68, CD31, HIF-1α, HIF-2α, VEGF (vascular endothelial growth factor), FR-α (folate receptor 1 or α), FR-β (folate receptor 2 or β), IL-8, MMP-9 (matrix metalloproteinase 9), Ang-1 (angiopoietin 1) and Ang-2 (angiopoietin 2). Ct (threshold cycle) values were determined with the software program SDS 2.3 (Applied Biosystems, Foster City, CA, USA). Samples that needed more then 30 cycles of GAPDH (reference gene) before reaching threshold were discarded. Relative gene expression was normalized to the expression of GAPDH and calculated by the following formula; Relative gene expression = 2^{\Delta Ct} (\Delta Ct = Ct gene of interest – Ct GAPDH). Furthermore, samples of non-atherosclerotic umbilical artery were used as a control.

Statistics
Values are presented as mean ± standard deviation. For comparison of fluorescence efficiency measurements in the IVIS spectrum, a two-tailed, paired student T-test was used for parametric distributions. Non-paired continuous variables with a non-parametric distribution were analyzed using the Mann-Whitney U-test. For correlations, Pearson’s and Spearman’s correlation coefficients were used when appropriate. A two-sided P value < 0.05 was considered statistically significant. Statistical tests were done with the Statistical Package for the Social Sciences (SPSS statistics version 16.0, SPSS inc®, Chicago IL, USA).
CHAPTER 6

Results

Patient Characteristics
A total of 17 men and three women with a mean age of $68.7 \pm 9.4$ years were included. Twenty carotid plaques were obtained during surgery. Patient characteristics are shown in Table 1.

Table 1. Baseline characteristics and risk factors for atherosclerosis

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men, n (%)</td>
<td>17 (85)</td>
</tr>
<tr>
<td>Age, years</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>BMI, kg/m2</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Smoking status, n (%)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>&gt; 1 pack a day, n (%)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>≤ 1 pack a day, n (%)</td>
<td>5 (62)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>18 (90)</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>152 ± 21</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>83 ± 11</td>
</tr>
<tr>
<td>Use of antihypertensives, n (%)</td>
<td>18 (90)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>12 (60)</td>
</tr>
<tr>
<td>Use of lipid-lowering drugs, n (%)</td>
<td>19 (95)</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>3.2 ± 1.1</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>1 (5)</td>
</tr>
</tbody>
</table>

Unless otherwise indicated, data are expressed as mean ± standard deviation; percentages between brackets.

Fluorescence Imaging and Validation of Folate-FITC Labeling
To validate the folate-FITC signal, CD11b cells were stained for FR-β. In isolated mononuclear cells from synovial fluid of a rheumatoid arthritis patient a large population of macrophages was found (63% were CD11b positive cells). Staining CD11b positive cells for FR-β shows the majority of the activated macrophages express FR-β (figure 1). Staining with folate-FITC gives a high percentage of positive cells, which can be blocked with free folic acid indicating that this is a specific interaction between FR-β and folate-FITC.
During fluorescence imaging of the plaques, using the IVIS spectrum®, the fluorescence signals reflecting the folate-FITC uptake occurred in specific areas of the plaques (Figure 2A). Fluorescence signals increased after 30 minutes of incubation with folate-FITC and reached a plateau phase after 30 minutes (Figure 2B). When this plateau was reached, the final images for comparison were taken. There was a heterogeneous distribution of fluorescence signals across the plaque's surface, both at the intraluminal and extraluminal surfaces. Mean background fluorescence (radiance, photons/sec/cm²/sr) as measured prior to incubation with folate-FITC was $1.5 \times 10^{-3} \pm 7.4 \times 10^{-4}$ intraluminally, and $2.5 \times 10^{-3} \pm 9.7 \times 10^{-4}$ extraluminally ($P<0.001$). After incubation with folate-FITC these values were $9.8 \times 10^{-3} \pm 2.6 \times 10^{-3}$ (intraluminally), and $5.5 \times 10^{-3} \pm 0.4 \times 10^{-3}$ (extraluminally) ($P<0.001$). Folate-FITC/background fluorescence ratios did not differ significantly between both sides of the plaque. There was a significant difference in intensity between background signals and folate-FITC intraluminally as well as extraluminally ($P<0.001$, Figure 2C).
**Figure 2.**

Figure 2. Ex vivo fluorescence imaging of human atherosclerotic plaques. (A) Fluorescence signals reflecting folate–FITC uptake occurring in specific areas of plaque intraluminally (upper) and extraluminally (bottom): incubation in phosphate-buffered saline (left), autofluorescence (middle), incubation with folate–FITC (right). (B) Intensity of fluorescence signals of plaques incubated with folate–FITC (solid bars) were increased both intraluminally and extraluminally when compared with autofluorescence signals (open bars). *P<0.001, **P<0.001. FR = folate receptor.

**Immunohistochemistry and Immunofluorescence**

Hot, medium and cold spots of 10 patients were stained with CD68 to identify macrophages in conjunction with folate–FITC uptake. Spots were identified as follows: cold spot exhibited low to no uptake; medium spot showed moderate uptake, and hot spot displayed high uptake of folate–FITC. Figure 3 shows a representative example of cold, medium and hot spots with respect to CD68 staining, along with quantification of the staining intensities at the cold, medium and hot spots.

**Figure 3.**

Figure 3. CD68 expression in atherosclerotic plaques of carotid arteries. (A) CD68 positive macrophages were nearly absent in a cold spot of such a plaque. (B) Extensive CD68 positive macrophage expression is present in a hot spot of a plaque. (C) The immunoreactivity score of CD68 was increased in hot spots (solid bar) versus cold spots (open bar) in 10 patients. (P=0.06)
In Figure 4 staining for HIF-1α in these identical spots is depicted. HIF-1α upregulation was significantly increased in the hot spot compared to the cold spot (P=0.01). As a control, expression of CD31-positive cells for the presence of endothelial cells was found heterogeneously on all plaque specimens and there was no difference between hot, medium or cold spots concerning expression of endothelial cells (P=0.89).

**Figure 4.**

**Figure 4.** HIF-1α expression in atherosclerotic plaques of carotid arteries. (A) A cold spot of a plaque showed mild or no hypoxia (B) Hot spot of a plaque showing extensive hypoxia. (C) The immunoreactivity score of hypoxia was increased in hot spots (solid bar) versus cold spots (open bar) measured in 10 patients (P = 0.01).

To demonstrate co-localization of macrophages and FR-β in hot spots double staining using immunofluorescence with CD68 and FR-β was performed. Indeed, substantial co-localization of CD68 and FR-β expression was encountered, demonstrating the presence of FR-β on the majority of macrophages in hotspots (Figure 5).

**Figure 5.**

**Figure 5.** Colocalization of FR-β and macrophages in hot spot of plaque from carotid artery. Blue = DAPI staining of nuclei; red (TRITC) = CD68 expression, macrophage marker; green (APC) = FR-β expression. Overlay of yellow represents colocalization of CD68 and FR-β.
mRNA Expression

In 15 snap-frozen biopsies of atherosclerotic plaques and 3 control artery samples mRNA levels were determined of FR-α and -β, CD68 and CD31 (Figure 6A) and of HIF-1α, HIF-2α, VEGF, MMP-9, Ang-1, Ang-2, and IL-8 (Figure 6B). As expected, expression of the tumor-specific FR-α was not detected in the biopsies. Compared to control tissue mRNA expression of FR-β, CD68, and MMP-9 was significantly increased compared to control tissue (P=0.02, P=0.01, and P=0.01 respectively).

Figure 6.

Figure 6. Relative expression of mRNA levels in 15 atherosclerotic plaques and three control artery samples. Quantitative reverse transcription polymerase chain reaction was used to determine relative expression of different genes in human atherosclerotic plaques (black dots) compared to control artery tissue (open dots). (A) mRNA expression of FR-β and CD68 was significantly increased in atherosclerotic tissue compared to control tissue (P=0.02 and 0.01 respectively) (B) mRNA expression of all inflammatory markers was higher in atherosclerotic tissue compared to control tissue.

FR-β expression moreover, significantly correlated with expression of the activated macrophage marker CD68 (r=0.53, P=0.045) (Figure 7). Also, CD68 expression showed a positive correlation with HIF-1α expression (r=0.55, P=0.034) and MMP-9 expression (r=0.63, P=0.021). CD31 expression was positively correlated with VEGF (r=0.66, P=0.007) and Ang-1 expression (r=0.63, P=0.039). No clear correlation could be found between FR-β and HIF1-α (r=0.07, P=0.13), nor between VEGF and Ang-1 (r=0.15, P=0.26).
Figure 7. Correlation between mRNA levels of different genes in 15 human atherosclerotic plaques and three control artery samples. CD 68 expression versus FR-β expression determined by rt-PCR. A significant correlation was observed between CD68 expression and folate receptor-β expression (P=0.045).
CHAPTER 6

Discussion

In this study we demonstrated high expression of folate receptor-β in plaques of patients with severe atherosclerotic disease using a novel methodology for mapping biomarker activity using optical folate-FITC imaging and confirmed our results histologically and by RT-PCR. Folate-FITC specifically binds to folate receptors and in this context to the FR-β, which is a marker for inflammation as is specifically expressed on activated macrophages (10, 12, 25). Moreover, the folate receptor-β on the cell surface of activated macrophages can bind folate-conjugated fluorophores with high affinity (12, 25). The significant difference between background fluorescence and folate-FITC uptake at both the intra- and extraluminal sides of the plaque might confirm the actual folate-FITC uptake in the plaque by ex-vivo incubation, which was found to be heterogeneously distributed. Furthermore, a difference between the number of activated macrophages in cold, medium and hot spots by folate-FITC guided mapping, respectively, could be detected, indicating increased inflammation in hot spots. However, this result was not significantly, which might be a result of the small group of patients used for this experiment (n= 10). In a previous study, an excess of macrophages over vascular smooth muscle cells was found in plaques vulnerable to rupture (26). In a study by Sluimer et al., the presence of macrophages correlated with hypoxia and levels of HIF-1α (24). Evidence of hypoxia in vascular disease is supported by in vivo detection of hypoxia in macrophage rich regions in rabbit atherosclerosis (6). These reports are supported by our findings on the HIF-1α stainings, which exhibited significant differential expression in the cold, medium, and hot spots, and correlated with the number of activated macrophages. This new approach of macrophage detection by folate-FITC labeling seems feasible to detect activated macrophages by their increased expression of folate receptor-β and is supported by double staining experiments (Figure 5). In addition to morphological and functional (duplex ultrasound) imaging, imaging of vulnerable plaques by means of activated macrophages might contribute to a better risk assessment and selection of patients at risk for ischemic stroke.

Activated macrophages have FR-β on their surface and produce MMP-9. Symptomatic plaques showed a high relative gene expression of MMP-9 (Figure 6B) and correlated with CD68 expression. This is in accordance with previous research, in which over-expression of MMP-9 in plaques had a strong association with unstable plaques, and with the presence of macrophages within these plaques (20, 27). Furthermore, analysis of the presence of MMP-9 protein by ELISA within excised carotid plaques revealed high MMP-9 protein mass in calcified segments at or near the carotid bifurcation and in segments with intraplaque hemorrhage (22). Since MMP-9 also can be visualized using the IVIS technique as shown earlier by our group, this could be combined with dual visualization of MMP-9 and FR-β, leading to a better assessment of inflammatory sites in vulnerable atherosclerotic plaques (22).

Endothelial cells produce VEGF and angiopoietins. The Ang-2 expression in the plaques
was relatively high compared to Ang-1. This is in agreement with previous findings, where Ang-1 induced formation of stable vessels, while Ang-2 destabilized the interaction between endothelial cells and their supporting cells (28). In the current study, CD31 correlated positively with VEGF and angiopoietin expression. As was expected, CD31 did not correspond with the folate receptor-β imaging since endothelial cells do not express folate receptors (Low, personal observations).

Fluorescence imaging using a folate-FITC optical contrast agent probe can visualize the distribution of activated macrophages, and might therefore be a good marker of inflammation and vulnerability of human atherosclerotic plaques. However, there are several limitations to this study. The number of carotid plaques was relatively small. Moreover, the IVIS Spectrum equipment used in the current ex vivo set-up cannot be applied for in vivo use in humans. FITc was used because this is currently approved for intravenous use in targeting the folate receptor alpha as a potential useful probe in oncology patients. Current applications for non-invasive optical imaging of fluorescent signals within a human carotid artery are limited by the use of fluorophores with emission wavelengths within the range of 450 tot 600 nm such as FITC, leading to a limited penetration depth. Therefore, sophisticated imaging devices such as multispectral optoacoustic tomography (MSOT) for imaging of near-infrared fluorophores like IRDye-CW800 conjugated to folate might solve this problem for future clinical applications. It is anticipated that such a device could also be used for clinical purposes for identification of high-risk vulnerable atherosclerotic plaques (29). We recently started with folate-\textsuperscript{99mTc} (kindly provided by prof. P.S. Low) imaging. The results look promising; therefore we believe folate-\textsuperscript{99mTc} might be a good compound for in vivo use in the future using the SPECT modality.

Furthermore, fluorescence imaging with the IVIS spectrum as it is used in the current set-up by definition is two-dimensional, i.e. a hot spot on the extraluminal side is not per se a hot spot on the intraluminal side. Future ex vivo 3D rendering and more sensitive quantification of picomolar concentrations using fluorescence molecular tomography (FMT), as described by Ntziachristos et al. might solve this problem and is currently under investigation (30).

In conclusion, high FR-β expression, as visualized by optical fluorescence imaging using folate-FITC, correlated with increased HIF-1α staining and a trend could be seen between high FR-β expression and CD68, representing activated macrophages. In addition, FR-β mRNA levels correlated with CD68, HIF-1α, and MMP-9 mRNA expression. Molecular imaging of the pathogenesis related to the vulnerability of atherosclerotic plaques might enable the clinician to make more substantiated therapeutic decisions in the treatment of patients with carotid artery disease in the future.
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