Brief Report

Targeting Chemokine Receptor CXCR4 and Translocator Protein for Characterization of High-Risk Plaque in Carotid Stenosis Ex Vivo

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Background and Purpose—This pilot study aims to demonstrate the feasibility of targeting molecular characteristics of high-risk atherosclerotic plaque in symptomatic and asymptomatic carotid stenosis (CS), that is, upregulation of the translocator protein (TSPO) and the chemokine receptor type 4 (CXCR4), by means of molecular imaging.

Methods—In a translational setting, specimens of carotid plaques of patients with symptomatic and asymptomatic CS obtained by carotid endarterectomy were analyzed for the presence of TSPO and CXCR4 by autoradiography, using the positron emission tomography tracers ^{18}F-GE180 and ^{68}Ga-Pentixafor and evaluated by histopathology. In addition, ^{68}Ga-Pentixafor positron emission tomography/computed tomography was performed in a patient with high-grade CS.

Results—Distinct patterns of upregulation of TSPO (^{18}F-GE180 uptake) and CXCR4 (^{68}Ga-Pentixafor uptake) were identified in carotid plaque by autoradiography. The spatial distribution was associated with specific histological hallmarks that are established features of high-risk plaque: TSPO upregulation correlated with activated macrophages infiltration, whereas CXCR4 upregulation also corresponded to areas of intraplaque hemorrhage. ^{68}Ga-Pentixafor uptake was significantly higher in plaques of symptomatic compared with asymptomatic CS. Clinical positron emission tomography revealed marked ^{68}Ga-Pentixafor uptake in carotid plaque of a patient with high-grade CS.

Conclusions—Clinical imaging of molecular signatures of high-risk atherosclerotic plaque is feasible and may become a promising diagnostic tool for comprehensive characterization of carotid disease. This methodology provides a platform for future studies targeting carotid plaque. **(*Stroke*. 2018;49:00-00. DOI: 10.1161/STROKEAHA.118.021070.)**

Key Words: autoradiography ■ carotid stenosis ■ carotid endarterectomy ■ CXCR4 ■ molecular imaging ■ TSPO

Carotid stenosis (CS) is an important cause of ischemic stroke. Currently, high-grade symptomatic CS is treated with carotid endarterectomy or percutaneous carotid stenting for secondary stroke prevention. However, indication for such intervention is challenging in asymptomatic CS because the benefit is controversial. Appropriate selection of patients at high-risk for stroke is needed, but reliable markers for risk stratification of CS are still lacking.

High-risk plaque is characterized by distinct histopathologic features, including a predominance of inflammatory cells, particularly activated macrophages, and intraplaque hemorrhage.[1,2] Recruitment of inflammatory cells to the atherosclerotic plaque is orchestrated via chemokines, such as monocyte chemoattractant protein-1 and CXCL12.[3,4] The CXCL12/chemokine receptor type 4 (CXCR4)-axis has emerged as a promising target for characterization of atherosclerotic plaque.[5,6] CXCR4 is a chemokine receptor expressed by a variety of inflammatory cells, including monocytes/macrophages, and other cells, such as platelets.[7,8] Elevated CXCR4-expression in atherosclerotic vessel wall compared with healthy arteries was recently reported.[9] Moreover, activated macrophages are characterized by marked expression of

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the translocator protein (TSPO).[10] Clinical molecular imaging has been recently used to noninvasively characterize TSPO[11] or CXCR4[5,12] in atherosclerotic plaque.

Therefore, we aimed to demonstrate the feasibility of combined targeting of specific molecular signatures of high-risk plaque in symptomatic and asymptomatic CS.

Materials and Methods

The authors declare that all supporting data are available within the article. Four patients with symptomatic (acute ischemic stroke [n=2], retinal artery occlusion [n=1], transient ischemic attack [n=1]) and 5 patients with asymptomatic CS were recruited at the Department of Neurology and at the Department of Cardiothoracic, Transplantation, and Vascular Surgery at Hannover Medical School, Germany. Patients with asymptomatic CS were admitted for surgery for primary stroke prevention after routine check-up ultrasound. All patients provided written informed consent. The study was approved by the local ethics committee.

Diagnostic workup, including Doppler/duplex-ultrasound of the brain supplying arteries, imaging studies of the brain and brain supplying arteries (computer tomography or magnetic resonance imaging), and transthoracic/trans esophageal echocardiography revealed CS as the cause of the described events in the patients diagnosed with symptomatic CS. Stroke severity was evaluated using the National Institutes of Health Stroke Scale. Cerebrovascular risk was rated using the Essen Stroke Risk Score.

Carotid plaques were obtained in the course of carotid endarterectomy and were stored snap frozen. For further analysis, the specimens were cryosectioned and mounted onto slides (14 µm; 3 slices per slide) with sequential slides assigned to autoradiography and histology.

To evaluate the expression of TSPO and CXCR4, in vitro autoradiography was performed in nonfixed carotid endarterectomy specimens using ^{18}F-GE180 and ^{68}Ga-Pentixafor, respectively. Tracers were synthetized as described previously.[5,13] Carotid artery sections were incubated for 30 minutes in 50 mmol/L PBS with ^{18}F-GE180 (19.40 kBq/mL) or ^{68}Ga-Pentixafor (24.25 kBq/mL) after 30-minute preincubation at room temperature in PBS. Slides were then washed with ice-cold PBS for 5 minutes and then rinsed in ice-cold distilled water 3× to remove buffer salts. Subsequently, slides were dried at room temperature. Once dried, slides were exposed to a high-resolution phosphor imaging plate (PerkinElmer, Downers Grove, IL) for 15 minutes and digitalized in a Cyclone scanner (PerkinElmer). A calibration curve was obtained by including a dilution series of known activity concentrations (range, 100–450 kBq/mL in 1.5 µL) on thin layer chromatography plates mounted to a microscope slide during each exposure. For analysis, digital images were inverted, and gray scale values were converted to Bq/mm^2 using the calibration curve. Tracer uptake was analyzed by quantifying the average concentration of the 300 hottest pixels of each of the 3 sections from each patient using PMOD software (Pmod Technologies, Zürich, Switzerland). Group average of symptomatic and asymptomatic carotid plaques was compared by 2-tailed Student *t* test and considered significant at $P<0.05$. Areas of different uptake of ^{18}F-GE180 and ^{68}Ga-Pentixafor underwent detailed analysis by hematoxylin-eosin histology. The histological analysis was performed by a pathologist (W.J. Schulz-Schaeffer) blinded to patients' characteristics and diagnosis.

In addition, 1 patient with stroke and concurrent etiologies (CS and atrial fibrillation) underwent clinical positron emission tomography after injection of 93 MBq of ^{68}Ga-Pentixafor, as previously described in a cohort with noncardiovascular indications for imaging.[5]

Results

Patients' characteristics are shown in the Table. Cerebrovascular risk was high in both groups with a median Essen Stroke Risk Score of 4.

^{18}F-GE180 and ^{68}Ga-Pentixafor showed marked, mainly focal uptake within carotid plaque specimens (Figure 1A and 1B). Distinct histological features were identified in focal uptake regions for the 2 tracers: high ^{18}F-GE180 uptake corresponded to activated macrophages in all specimens. By contrast, ^{68}Ga-Pentixafor uptake correlated mainly with areas of intraplaque hemorrhage.

There was no significant difference of ^{18}F-GE180 uptake between symptomatic and asymptomatic patients (316.2±151.4 Bq/mm^2 [mean±SD; N=4] versus 492.9±157.5 Bq/mm^2 [mean±SD; N=5]; $P=0.1329$; Figure 1C). By contrast, comparison of ^{68}Ga-Pentixafor uptake between symptomatic and asymptomatic carotid plaque (8.01±3.15 Bq/mm^2 [mean±SD; N=4] versus 4.21±1.52 Bq/mm^2 [mean±SD; N=5]) passed the level of significance ($P=0.0475$; Figure 1D).

Clinical molecular imaging by positron emission tomography demonstrated marked ^{68}Ga-Pentixafor uptake in CS (Figure 2), which was considerably higher than the contralateral carotid artery (SUV_{max}, 2.9 versus 1.8).

Discussion

Targeting TSPO and CXCR4 in CS is feasible and provides distinct information concerning plaque composition. Uptake of ^{18}F-GE180 (TSPO) and ^{68}Ga-Pentixafor (CXCR4) corresponded to histological hallmarks of high-risk plaque. Moreover, CXCR4-expression was higher in symptomatic than in asymptomatic carotid plaque specimens as determined by ^{68}Ga-Pentixafor autoradiography.

Recently, the feasibility of clinical CXCR4 imaging in atherosclerotic plaques was demonstrated,[5] and reports of CXCR4–positron emission tomography of carotid arteries of rabbits and humans showed a suitable ^{68}Ga-Pentixafor uptake in the diseased vessel wall.[5,6] The importance of the CXCL12/CXCR4 axis in atherosclerosis is further supported by several clinical and experimental studies. For example, CXCR4 expression by circulating progenitor cells was significantly

Table. Patients' Characteristics

Group	Symptomatic	Asymptomatic
N	4	5
Age, y (median±SD)	77.90 (±6.11)	77.83 (±6.54)
Sex (male/female)	2/2	3/2
ESRS (median±SD)	4 (±1.26)	4 (±1.30)
Stenosis grade		
Moderate	1 (25%)	0
Severe	1 (25%)	5 (100%)
Highly severe	2 (50%)	0
Initial NIHSS (median±SD)	2 (±3.40)	N/A
Intravenous r-tPA therapy	2 (50%)	N/A

Stenosis grade was determined according to NASCET criteria. Moderate stenosis: 50% to 70%; severe stenosis: 70% to 90%; highly severe stenosis: >90%. ESRS indicates Essen Stroke Risk Score; N/A, not applicable; NASCET, North American Symptomatic Carotid Endarterectomy Trial; NIHSS, National Institutes of Health Stroke Scale; and r-tPA, reverse tissue-type plasminogen activator.

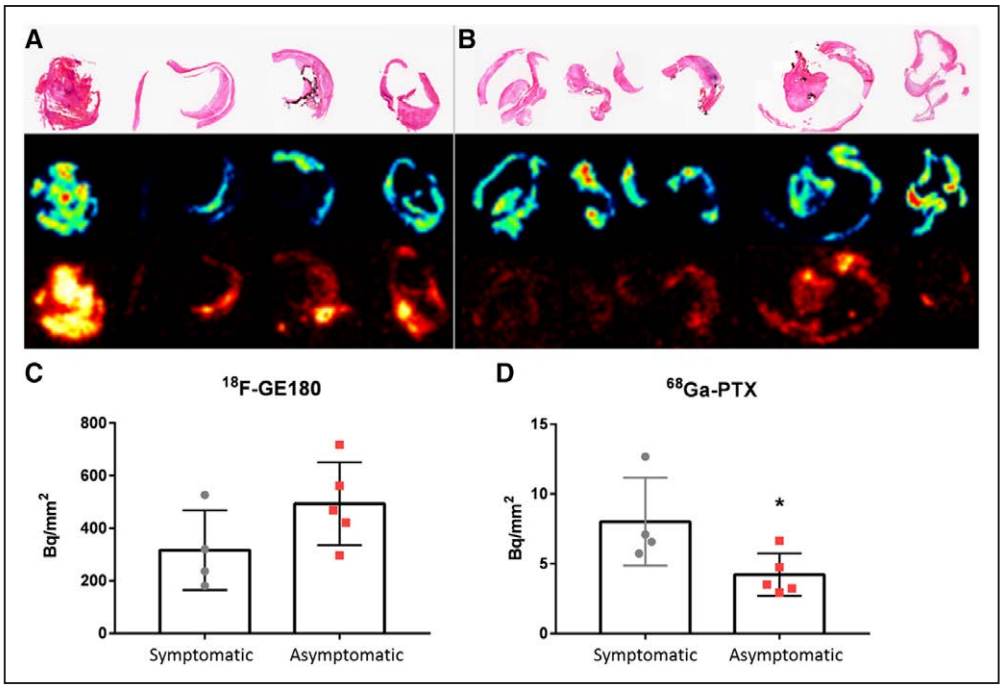

Figure 1. Hematoxylin-eosin (H&E) stain and autoradiography of TSPO (translocator protein) and chemokine receptor type 4 (CXCR4) expression using ^{18}F-GE180 and ^{68}Ga-Pentixafor (^{68}Ga-PTX) in carotid artery plaques. Distinct uptake patterns of ^{18}F-GE180 and ^{68}Ga-PTX in symptomatic (**A**) and asymptomatic (**B**) carotid artery plaques. First line (**A** and **B**): H&E, second line: ^{18}F-GE180, third line: ^{68}Ga-PTX. Quantification of ^{18}F-GE180 (**C**) and ^{68}Ga-PTX (**D**) uptake in symptomatic and asymptomatic carotid artery plaques by autoradiography (Bq/mm^2), mean values±SD.

higher in patients with an unstable carotid plaque compared with those with a stable one, as evaluated using magnetic resonance imaging, including consideration of intraplaque hemorrhage.[14] In addition to inflammation, neovascularization and intraplaque hemorrhage are established features of high-risk atherosclerotic plaque.[1] Importantly, ^{68}Ga-Pentixafor uptake was associated with intraplaque hemorrhage, wherein the highest autoradiography signal was observed in a carotid plaque of a symptomatic patient. Platelets express CXCR4[8]. Hypothetically, ^{68}Ga-Pentixafor uptake may thus also be linked to platelet accumulation in areas of hemorrhage, besides expression by inflammatory cells, as described previously.[6] However, histological analysis revealed infiltration of macrophages and other inflammatory cell populations which additionally contributed to a ^{68}Ga-Pentixafor signal. Future studies investigating ^{68}Ga-Pentixafor as potential imaging marker of atherosclerotic plaque vulnerability should, therefore, include immunohistochemistry for direct comparison of tracer uptake with distinct cellular and molecular characteristics. The uptake of ^{18}F-GE180 was clearly associated with macrophage-rich areas, consistent with previous TSPO studies.[10] However, in this pilot study, no significant difference in ^{18}F-GE180 uptake was observed between symptomatic and asymptomatic patients. Importantly, so far, asymptomatic CS may also feature hallmarks of high-risk plaque, and TSPO imaging may thus be valuable for early identification of patient risk.

The presented approach may be used to comprehensively characterize atherosclerotic carotid disease and bears promise for identification of high-risk plaque in CS. In this study, ^{68}Ga-Pentixafor uptake at autoradiography was significantly higher in symptomatic compared with asymptomatic carotid plaques. Translation of this approach to a clinical setting demonstrated markedly higher uptake in high-grade CS compared with the contralateral artery.

The main limitation of the current pilot study is the small sample size, such that statistics should be interpreted with caution. While we did not perform double staining using immunohistochemistry, for example, regarding platelets or chemokine receptors, we nevertheless identified distinct patterns of ^{68}Ga-Pentixafor (CXCR4 imaging biomarker) and ^{18}F-GE180 uptake (imaging biomarker for activated microglia/macrophages) which correlated to established histological hallmarks of high-risk carotid plaque. These results support

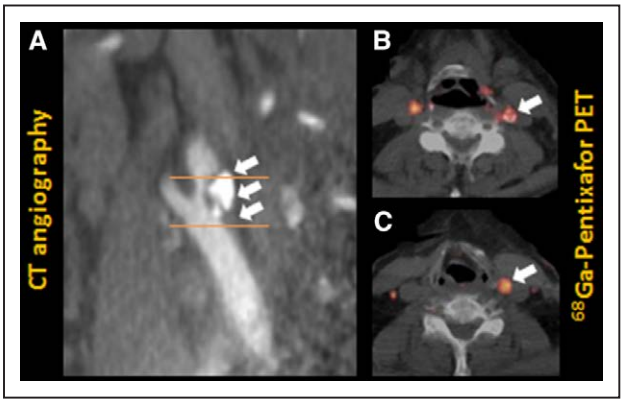

Figure 2. In vivo computed tomography (CT) angiography and ^{68}Ga-Pentixafor positron emission tomography (PET) in a patient with high-grade carotid stenosis. CT angiography identifies high-grade carotid stenosis due to partly calcified plaque (**A**). Upper calcified portion exhibits elevated ^{68}Ga-Pentixafor uptake, similar to contralateral atherosclerotic vessel wall (**B**). Tracer uptake in the noncalcified plaque area (**C**) is markedly higher than in the contralateral artery. Arrows indicate plaque. Orange lines indicate intersections.

further (in vivo) studies evaluating the 2 tracers as potential markers for risk stratification in CS.

Conclusions

In this pilot study, we demonstrated the feasibility of targeting CXCR4 and TSPO-expression in human carotid plaque specimens. Molecular imaging of markers of high-risk carotid plaque may be promising as a diagnostic tool. Larger clinical studies combining histopathology, immunohistochemistry, and in vivo-imaging are warranted. This multifaceted approach provides a platform for such future studies targeting CS.

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Disclosures

Dr Bengel reports research support from GE Healthcare. S. Kropf is the chief executive officer of SCINTOMICS GmbH, Germany. Dr Wester is a shareholder of SCINTOMICS GmbH, Germany. SCINTOMICS owns the IP on Pentixafor. The other authors report no conflicts.

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