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Article

Evaluation of the Radiolabeled Boronic Acid-Based FAP Inhibitor MIP-1232 for Atherosclerotic Plaque Imaging

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Abstract: Research towards the non-invasive imaging of atherosclerotic plaques is of high clinical priority as early recognition of vulnerable plaques may reduce the incidence of cardiovascular events. The fibroblast activation protein alpha (FAP) was recently proposed as inflammation-induced protease involved in the process of plaque vulnerability. In this study, FAP mRNA and protein levels were investigated by quantitative polymerase chain reaction and immunohistochemistry, respectively, in human endarterectomized carotid plaques. A published boronic-acid based FAP inhibitor, MIP-1232, was synthetized and radiolabeled with iodine-125. The potential of this radiotracer to image plaques was evaluated by *in vitro* autoradiography with human carotid plaques. Specificity was assessed with a xenograft with high and one with low FAP level, grown in mice. Target expression analyses revealed a moderately higher protein level in atherosclerotic plaques than normal

arteries correlating with plaque vulnerability. No difference in expression was determined on mRNA level. The radiotracer was successfully produced and accumulated strongly in the FAP-positive SK-Mel-187 melanoma xenograft *in vitro* while accumulation was negligible in an NCI-H69 xenograft with low FAP levels. Binding of the tracer to endarterectomized tissue was similar in plaques and normal arteries, hampering its use for atherosclerosis imaging.

Keywords: atherosclerosis; fibroblast activation protein; carotid artery plaque; boronic acid-based inhibitor

1. Introduction

The concept of plaque vulnerability has changed the understanding of the pathogenesis of atherosclerosis and has led to novel perspectives for diagnostic and therapeutic interventions. The development of diagnostic methods to assess plaque vulnerability is considered an urgent priority in clinical and basic research [1]. The assessment of plaque vulnerability in patients at risk for cardiovascular disease would allow an adequate pharmacological and/or surgical treatment already in the asymptomatic stage and, therefore, reduce atherosclerosis-associated disability and mortality. Molecular imaging with suitable tracers has the potential to non-invasively identify molecular processes providing functional information about disease progression. In the asymptomatic stage, functional imaging may thus provide more specific information on plaque vulnerability than morphology-based imaging modalities [2]. Several imaging targets and the respective tracers are under investigation with the goal to image plaque vulnerability. The most prominent tracer is [^{18}F]fluorodeoxyglucose, which accumulates in cells with high glucose consumption, including activated macrophages. However, the unspecific mechanisms of accumulation and the high uptake in myocardium limit its applicability [3].

Nowadays, plaque progression is regarded as a dynamic and complex process with stabilizing and destabilizing components involved. If destabilizing plaque components prevail over stabilizing factors an atherosclerotic plaque may eventually rupture leading to often severe or even fatal complications. Stabilizing components include an intact and thick fibrous cap that is formed by smooth muscle cells (SMCs) embedded in an extracellular matrix rich in collagen. On the contrary, plaque vulnerability is related to a thinning of the fibrous cap facilitated by the gradual loss of SMCs and the degradation of the collagen-rich fibrous cap [4]. The digestion of the extracellular matrix is caused by proteases in the atheromata which include matrix metalloproteinases (MMPs), cathepsins S/K and as recently proposed the fibroblast activation protein alpha (FAP, seprase) [5–8]. FAP is a type II membrane-bound serine protease belonging to the subfamily dipeptidyl peptidase IV N-terminal (DPP IV, S9B) within the prolyl oligopeptidase family (POP, S9) [9–11]. In contrast to other members of the DPP IV subfamily, FAP displays endo- besides exopeptidase activity [12]. FAP is capable of cleaving peptide bonds between proline and another amino acid [12]. FAP has gelatinase activity and is involved in the further digestion of degradation products of type I collagen [13–16]. The endo- and exopeptidase enzymatic activity requires homodimerization and glycosylation of the protease [10,14,17].

FAP was initially identified as a pivotal component of the tumor microenvironment expressed by reactive stromal fibroblasts in over 90% of common human epithelial carcinomas and may serve as a

therapy target in oncology [18–20]. Furthermore, an association of FAP expression with inflammatory processes was described [18] and in line with this finding is emerging data by Brokopp *et al.* indicating an involvement of FAP in the pathogenesis of atherosclerosis [7]. In detail, Brokopp *et al.* showed that FAP is expressed by SMCs in human aortic plaques and confirmed its involvement in type I collagen degradation in aortic fibrous caps. Moreover, an association between tumor necrosis factor alpha (TNFα) secretion by macrophages with FAP expression in cultured human aortic SMCs and additionally a positive correlation of FAP-expressing SMCs with the macrophage burden in human aortic plaques was described [7]. The extent of FAP expression at different stages in atherosclerotic plaque progression was evaluated and revealed an increased FAP expression in advanced aortic plaques and in thin-cap *versus* thick-cap coronary atheromata by immunohistochemistry and immunofluorescence [7]. These findings indicate that FAP expression is related to plaque vulnerability with FAP representing an inflammation-induced protease in atherosclerosis. In this respect, FAP could serve as a promising target for non-invasive atherosclerotic plaque imaging.

The goal of this study was to evaluate FAP as a target for atherosclerosis imaging with a small molecule. Imaging FAP density requires a FAP-selective ligand with high binding affinity. Several research groups have pursued to design small inhibitors with high specificity and selectivity towards individual serine proteases in the POP family. To selectively target FAP over other peptidases, its dual enzymatic activity as endo- and exopeptidase has to be considered. Identifying inhibitors with high selectivity for FAP over other DPPs and the most closely related prolyl endopeptidase PREP is challenging due to the 48% amino acid sequence identity of FAP and DPP-4, analogous substrate preferences and the ubiquitous expression of many proteases of the POP family [9,11]. Most FAP inhibitors share the pyrrolidine-2-boronic acid moiety as a common structural motif. The first boronic acid inhibitor reaching phase II clinical trials in the field of cancer treatment was ValboroPro (talabostat, PT-100), however due to missing selectivity clinical evaluation was terminated [21–23]. ValboroPro displayed IC_{50} values in the nanomolar range to several prolyl peptidases [24]. The introduction of a blocked N terminus in the dipeptidyl boronic acid structure led to novel inhibitors that were evaluated regarding binding affinity and selectivity [25–29] with the advantage of impeded intra-molecular cyclization reactions mediated by the electrophilic boron and an increased selectivity over DPPs that lack endopeptidase activity [30].

Marquis *et al.* presented a para-iodine substituted benzamido-glycine-boronoproline analog, MIP-1232, with an IC_{50} of 0.6 nM as determined in an enzyme inhibition assay with human recombinant FAP [29]. MIP-1232 was 32-fold more potent in inhibiting FAP than PREP. The corresponding K_d value of [^{123}I]MIP-1232 in stably FAP-transfected human embryonic kidney cells (HEK-293) was 30 nM and different FAP-positive cell lines showed a markedly reduced enzymatic activity under MIP-1232 treatment compared to baseline conditions [29,31]. The high binding affinity to FAP and the selectivity profile in combination with the possibility to radioiodinate MIP-1232 without altering its structure make this compound a promising molecule to assess the potential of FAP as an imaging target for the staging of plaque vulnerability and to detect FAP-positive tumors that may respond to FAP-targeted therapy. In this study, we investigated FAP expression in human carotid specimens by quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC). Furthermore, we synthesized MIP-1232 and subsequently radiolabeled this compound with iodine-125. Its accumulation in human atherosclerotic plaques was evaluated *in vitro* by autoradiography.

A FAP-positive SK-Mel-187 melanoma xenograft and an NCI-H69 xenograft with low FAP levels, both grown in mice, were used as controls.

2. Results and Discussion

2.1. Gene Expression Analysis of FAP and SMA in Human Carotid Plaques

Quantitative expression analysis of FAP and alpha smooth muscle cell actin (SMA) by qPCR was performed with β-actin as reference gene (Figure 1). For FAP, a similar average gene expression was determined in normal arteries, stable plaques and vulnerable plaques (Figure 1A). The average SMA gene expression was not significantly different comparing vulnerable and stable plaques (Figure 1B). No significant correlation between the SMA and FAP gene expression in human endarterectomized plaques was observed (Figure 1C).

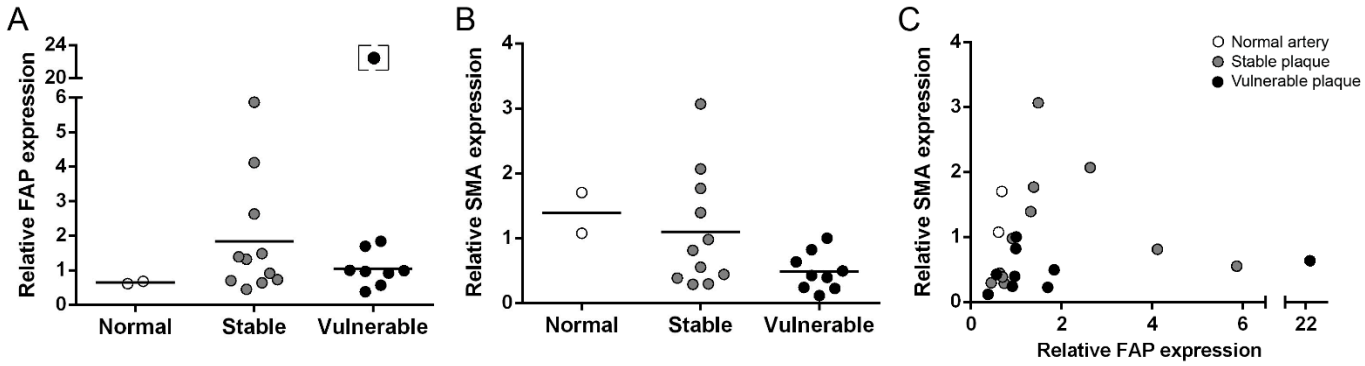

Figure 1. Relative mRNA expression levels of FAP (**A**) and SMA (**B**) in normal arteries (n = 2), stable plaques (n = 11) and vulnerable plaques (n = 9). For both proteins no significant difference was detected between stable and vulnerable plaques. (**C**) Comparison of the relative mRNA expression levels of FAP and SMA. mRNA expression was quantified by qPCR, shown are averages of three independent analyses. Lines indicate mean values. The square bracket indicates an outlier that was excluded from statistical analyses.

2.2. Immunohistochemical Staining of Human Carotid Plaques for FAP and SMA

The expression of FAP and SMA was further investigated by immunohistochemistry in consecutive sections of human atherosclerotic plaques (Figure 2). Normal arteries were FAP negative. In plaques, a focal FAP expression in macrophages and giant cells located in the superficial regions of the fibrous cap was observed with the most pronounced focal signals in vulnerable plaques (Figures 2C2,D1,D2). SMA was strongly expressed in the tunica media in all three classification categories with the highest expression in the vasa vasorum of normal arteries (Figure 2A1). The distribution pattern of SMA within atherosclerotic plaques was generally focal with major clusters in the cap or shoulder region.

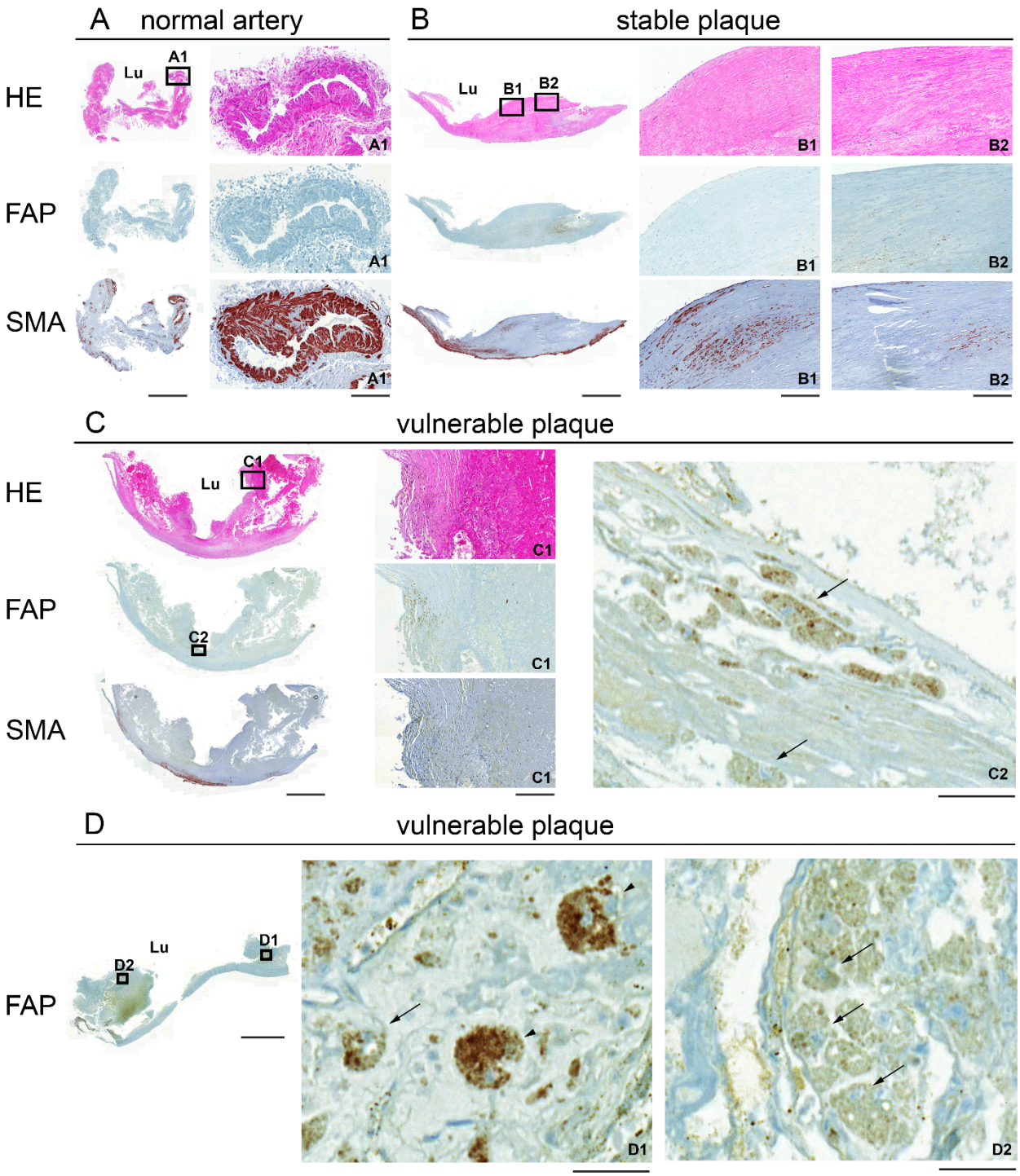

Figure 2. Hematoxylin/eosin (HE; **A**–**C**) and immunohistochemical (**A**–**D**) staining for FAP and SMA of representative 2 µm paraffin-embedded sections of a normal artery (A), a stable plaque (B) and vulnerable plaques (C,D). Boxed higher-magnification images show a small blood vessel (normal artery A1), regions in the fibrous cap (stable B1, B2 and vulnerable plaque C1) and FAP-positive macrophages (C2, arrows). (D) High magnification images show FAP-positive giant cells (D1, arrowheads) and macrophages (D1, D2, arrows) in a vulnerable plaque. The endarterectomized plaques are composed of tunica intima and part of the media. Lu: lumen. Scale bar, low magnification 2000 µm; A1, B1, B2, C1, 200 µm; C2, D1, D2, 50 µm.

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