Loss of protease activity of ADAM15 abolishes protective effects on plaque progression in atherosclerosis

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The A Disintegrin And Metallporteinases (ADAMs) contain a metalloprotease-like and a disintegrin-like domain. Currently 40 different types of ADAM proteins have been identified. ADAM15 is found in the myocardium [1,2], endothelial cells and in vascular atherosclerotic lesions [3]. ADAM15 expression is not only regulated in different disease states such as in human dilated cardiomyopathy [1] and atrial fibrillation [2] but also during progression of atherosclerosis [4]. ADAM15 has been described as a mediator of inflammation via the RGD sequence, the binding domain for the integrins α_5/β_1, α_v/β_3 [5] and α_{IIb}/β_3 [6]. On the other hand, the exact function and particularly the substrates for the protease of ADAM15 have not yet been identified.

In order to characterize the role of ADAM15 in atherosclerosis we evaluated ADAM15 in a recently established rabbit model for target validation in atherosclerosis that allows the evaluation of morphology and endothelial dysfunction [7]. Additionally, a mutated ADAM15 with ablated protease activity ("ADAM15 prot neg") was investigated.

To generate an adenovirus coding for ADAM15 or a mutant with ablated protease activity, a PCR cloning technique was used. For Ad-ADAM15, the forward primer gcgggggcggccgcccaccatgcggctggcgctgctctgg and reverse primer gcggggtctagatcacttgtcgtcgtcgtccttatagtcgaggtagagc-gaggacactgtcg were used. For adenovirus coding for ADAM15 with ablated protease activity (Ad-ADAM15 prot neg), the primer pairs cctccatagcc-catcagttgggccacagcctgggc and gtggcccaactgatgggctatggaggaggcgac were used for overlapping PCR to mutate amino acid E at position 349 to Q within the zinc-binding consensus sequence or catalytic consensus sequence (HEXXH). This point mutation has been previously described to completely ablate function in metalloproteases [8,9] or endopeptidases [10,11]. Recombinant adenovirus was cloned and generated after recombination in *E. coli* BJ5183 and transfection and amplification in HEK 293 cells as previously described [7]. A control adenovirus containing the reporter gene GFP (AdGFP) only was always used for respective controls.

For the induction of atherosclerosis, rabbits were fed with Western type high cholesterol (0.25%) diet for 8 weeks and vascular gene transfer to the carotid artery was performed as previously described [7].

Animals were sacrificed 4 weeks after the adenovirus delivery. The left common carotid arteries, aorta and iliac arteries were macroscopically prepared for "en face" evaluation of plaque extension and stained with Sudan III. Serial 6-μm-thick cryosections were cut and histological assessment of atherosclerosis after hematoxylin eosin (HE) and van Gieson (VG)-elastica staining and GFP expression were performed. Immunohistochemical analysis, with anti rabbit RAM 11 antibody (DAKO, Hamburg, Germany) was used for macrophages as previously described [12].

After vascular gene transfer into the carotid artery, GFP expression could be detected with AdGFP and also with Ad-ADAM15 and Ad-ADAM15 prot neg, which both co-expressed GFP (Fig. 1). Relative plaque size was significantly reduced in the Ad-ADAM15 infected rabbits compared to the AdGFP controls, but not with Ad-ADAM15 prot neg (see macroscopic preparations in Fig. 2A and B and detailed histology after HE and VG staining in Fig. 2C and D). Macrophage density was also significantly reduced with Ad-ADAM15 but not with Ad-ADAM15 prot neg compared to GFP control (see Fig. 2E and F). In vivo endothelial function in cholesterol-fed rabbits was compromised compared to healthy littermates (compare Fig. 3A with Fig. 3B). However, Ad-ADAM15 and Ad-ADAM15 prot neg had no significant effect on endothelial dysfunction in atherosclerotic rabbits (see Fig. 3A). In contrast Ad-ADAM15 and Ad-ADAM15 prot neg led to a significant reduction of vascular dilatation in healthy animals (see Fig. 3B).

In this study, we demonstrate the physiological significance of ADAM15 both in healthy and in atherosclerotic endothelium in vivo. ADAM15 attenuated the progression of atherosclerosis in cholesterol-fed rabbits. In contrast, a mutated form of ADAM15 – which was created by ablation of the protease function – had no significant effect on atherosclerosis. Endothelial mediated vasoreactivity was impaired by both, mutant and wild type ADAM15 in healthy animals. Interestingly, the improvement in the morphology of atherosclerosis does not result in improved endothelial function in atherosclerotic rabbits.

It is surprising that ADAM15 overexpression in the vascular endothelium results in a beneficial overall effect on the development of atherosclerosis. Previous authors described interactions of the RGD binding site of ADAM15 with α_5/β_1; α_v/β_3 [5] and α_{IIb}/β_3 [6]. Thus a potential role for ADAM15 as a ligand for integrins on platelets promoting atherosclerosis via increased platelet adhesion and secretion of pro-inflammatory cytokines (CD40L; P-Selectin) was suggested [13].

The present data are the first to investigate the role of ADAM15 on atherosclerosis in vivo. Here ADAM15 with intact protease function seems to have a strong beneficial effect on atheroprogression. Mainly, the intact protease function seems to be essential for this effect. The substrate for the ADAM15 protease has not yet been identified. However, ADAM 17 (TACE) cleaves the chemokine fractalkine, which functions as an adhesion molecule on endothelial cells and binds to its receptor, CX3CR1, on the surface of monocytes [14]. Moreover, cleavage of L-Selectin [15], or the

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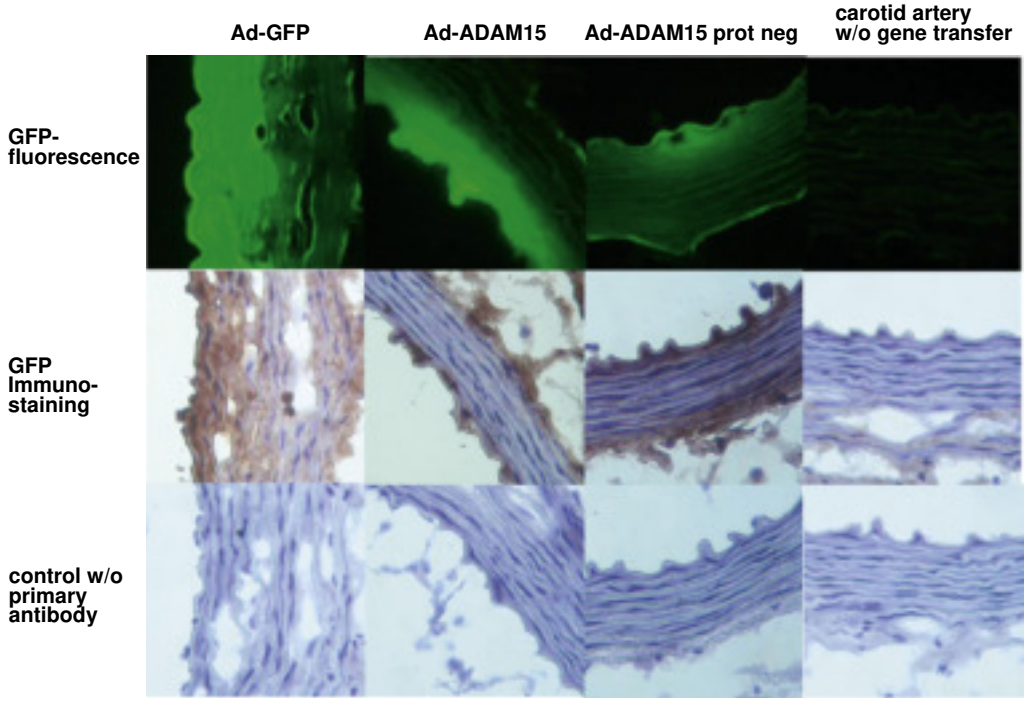

Fig. 1. Expression of the reporter gene GFP was assessed in carotid arteries after gene transfer in rabbits in vivo. 4 weeks after gene transfer with Ad-ADAM15 (coexpressing GFP with ADAM15), Ad-ADAM15 prot neg (coexpressing GFP with ADAM15 with ablated metalloprotease activity) and Ad-GFP, GFP expression was detected in all rabbits. In representative histological sections GFP fluorescence was viewed in the carotid artery (upper panel). As comparison, fluorescence was viewed in the carotid artery of rabbits without local vascular gene transfer. With specific anti-GFP immunohistochemistry a similar pattern of GFP expression could be detected (middle panel). The specificity of GFP immunostaining is verified by the negative control without the specific primary anti GFP antibody (lower panel).

vascular adhesion molecule 1 (VCAM-1) [16] has been described for ADAM 17, which both play an important role in cell–cell interactions of monocytes to activated vascular endothelium during the progression of atherosclerosis. Thus the protective effects of ADAM15 in the present study would be in line with cleavage of adhesion molecules previously described for other ADAMs to ameliorate atherosclerosis.

In further experiments in healthy rabbits with normal endothelial function, somatic gene transfer of both, wild type ADAM15 and the mutated form of ADAM15, largely blunted endothelium-mediated vasodilatation. It is generally accepted, that endothelium dysfunction is mediated – among others – by decreased NO availability or increased NO degradation [17,18]. NO bioavailability is regulated by production via the endothelial NO synthase and degradation via increased reactive oxygen species (ROS). Platelets itself can produce huge amounts of ROS e.g. during arterial thrombosis (for review see [19]). Increased platelet adhesion and activation via the disintegrin site of ADAM15 as described by Langer [6] could therefore contribute to NO dependent endothelial dysfunction. Thus the two functional sites of ADAM15 seem to have contrary effects on morphology and the endothelial function during atheroprogression.

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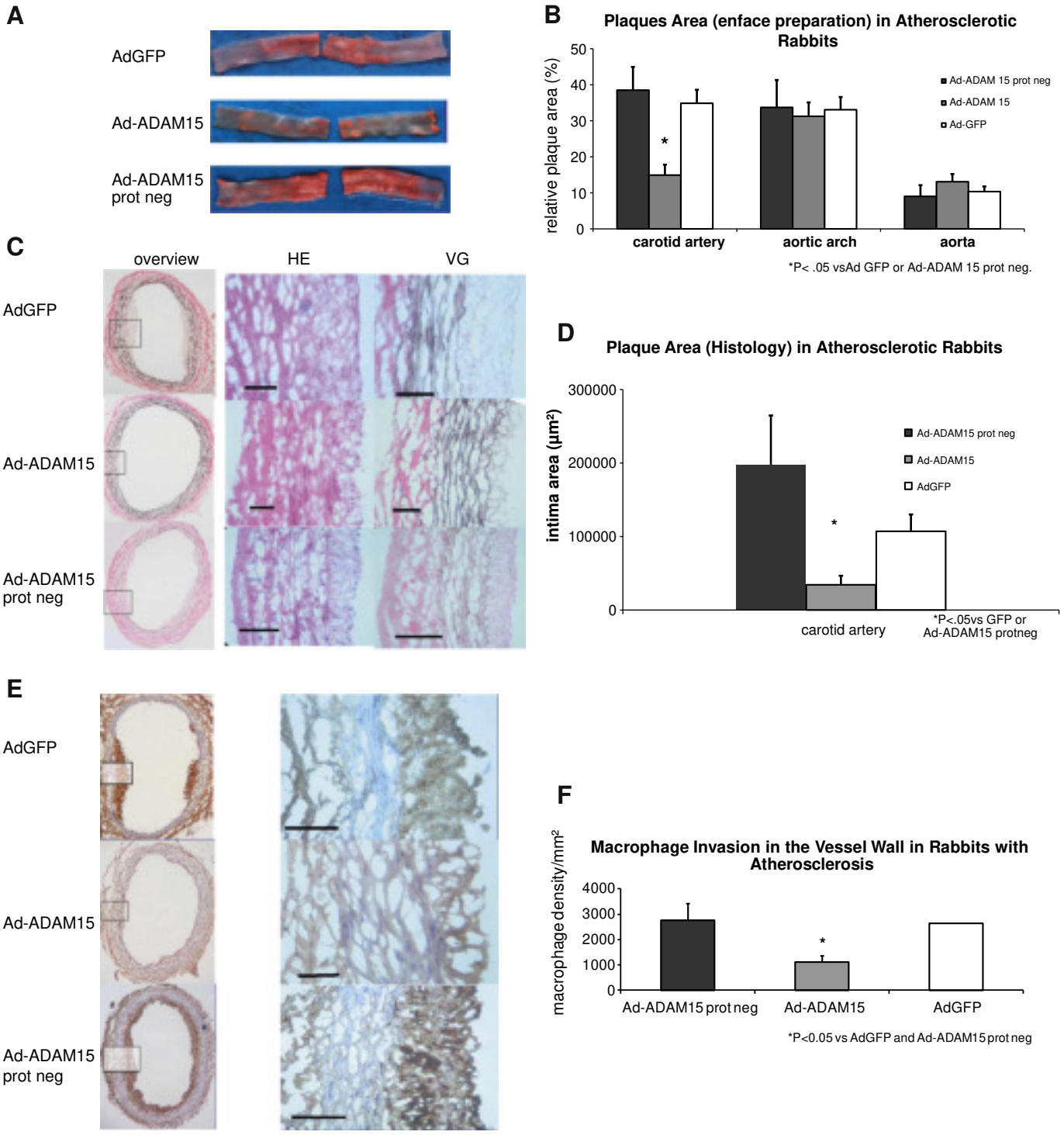

Fig. 2. (A) 4 weeks after local gene transfer in atherosclerotic rabbits' fatty streak plaque formation was reduced in the carotid artery after adenoviral gene transfer with Ad-ADAM15 compared to GFP controls (Ad-GFP; en face macroscopic preparations). The mutated form of ADAM15 with ablated metalloprotease activity (Ad-ADAM15 prot neg) had no effect on fatty streak formation compared to GFP controls (Ad-GFP). (B) Quantitative assessment of fatty streak formation by digital image analysis showed significant reduction after local gene transfer with Ad-ADAM15, but not with the Ad-ADAM15 prot neg. Other vascular sites without gene transfer served as internal controls demonstrating homogenous plaque progression in rabbits after high cholesterol diet. The means ± SEM of 7 animals are shown (* indicates $p<0.05$ significance compared to AdGFP). (C) In histological slices of the carotid artery, significant plaque formation in GFP-infected cholesterol-fed rabbits (Ad-GFP) was visible with haematoxylin eosin (HE) and elastica vanGieson (VG) staining. Local Ad-ADAM15 infection of the carotid artery resulted in reduced plaque formation, whereas Ad-ADAM-15 prot neg had no influence on plaque progression (D) Quantitative assessment of plaque size by digital image analysis showed significant reduction after local gene transfer with Ad-ADAM15, but not with the Ad-ADAM15 prot neg. (E) Invasion of macrophages into the atherosclerotic plaque is visualized by brown staining using immunohistochemistry. (F) Quantitative assessment demonstrated a significant reduction of macrophage invasion after local Ad-ADAM15 but not after Ad-ADAM15 prot neg gene transfer (* indicates $p<0.05$ significance compared to AdGFP). The means ± SEM of 7 animals are shown.

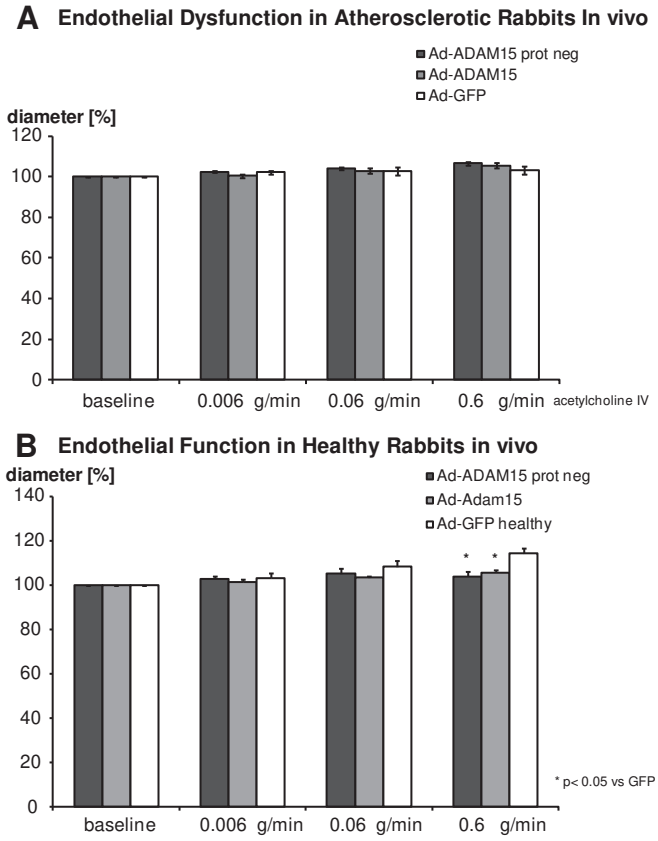

Fig. 3. (A) Endothelial dysfunction was investigated in rabbits by vascular ultrasound in vivo by infusion of increasing doses of acetylcholine. The effect of Ad-ADAM15, Ad-ADAM15 prot neg and control adenovirus (AdGFP) was compared in atherosclerotic rabbits. The relative change of the diameter of the carotid artery compared to the baseline diameter (in %) is demonstrated (* indicates $p<0.05$ significance compared to AdGFP). (B) The effect of Ad-ADAM15 and Ad-ADAM15 prot neg on endothelial function was also investigated in healthy rabbits without any sign of atherosclerosis. Both Ad-ADAM 15 and Ad-ADAM15 prot neg significantly impaired acetylcholine induced, endothelium-dependent vasoreactivity in healthy rabbits. (* indicates $p<0.05$ significance compared to AdGFP healthy). The means ± SEM of 7 animals are shown.

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A classification of bifurcation restenosis

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Coronary bifurcations are among the most complex lesions encountered in everyday interventional practice and are associated with a poorer outcome than simpler lesions.

The complexity of bifurcation lesions is mainly linked to a high variability in their anatomical pattern, with uneven involvement of the proximal and distal portions of the main vessel (MV) and/or the side branch (SB). This aspect has been the object of several classification proposals that are difficult to memorize [1–5] and an intuitive one, the Medina classification [6], that has gained wide consensus, owing to its simplicity.

After the introduction of a variety of bifurcation-specific techniques, as well as drug-eluting stents (DES), the high restenosis rate impairing the results of percutaneous coronary intervention (PCI) targeting bifurcations has considerably reduced [7].