Vascular Biology, Atherosclerosis and Endothelium Biology

T-Cell Activation Leads to Reduced Collagen Maturation in Atherosclerotic Plaques of *Apoe*^{-/-} Mice

Olga Ovchinnikova,^{*,†} Anna-Karin L. Robertson,^{*} Dick Wågsäter,^{*} Eduardo J. Folco,[‡] Marjo Hyry,[§] Johanna Myllyharju,[§] Per Eriksson,^{*} Peter Libby,[‡] and Göran K. Hansson^{*}

From the Center for Molecular Medicine and the Department of Medicine at Karolinska University Hospital,^{*} Karolinska Institute, Stockholm, Sweden; the Department of Internal Medicine No. 1,[†] Pavlov State Medical University, St. Petersburg, Russia; the Department of Medicine,[‡] Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts; and the Oulu Centre for Cell-Matrix Research, Biocenter Oulu and the Department of Medical Biochemistry and Molecular Biology,[§] University of Oulu, Oulu, Finland

Rupture of the collagenous, fibrous cap of an atherosclerotic plaque commonly causes thrombosis. Activated immune cells can secrete mediators that jeopardize the integrity of the fibrous cap. This study aimed to determine the relationship between T-cell-mediated inflammation and collagen turnover in a mouse model of experimental atherosclerosis. Both *Apoe*^{-/-} × CD4dnTβRII mice with defective transforming growth factor-β receptors in T cells (and hence released from tonic suppression of T-cell activation) and lesion size-matched *Apoe*^{-/-} mice were used. Picrosirius red staining showed a lower content of thick mature collagen fibers in lesions of *Apoe*^{-/-} × CD4dnTβRII mice, although both groups had similar levels of procollagen type I or III mRNA and total collagen content in lesions. Analysis of both gene expression and protein content showed a significant decrease of lysyl oxidase, the extracellular enzyme needed for collagen cross-linking, in aortas of *Apoe*^{-/-} – CD4dnTβRII mice. T-cell-driven inflammation provoked a selective and limited increase in the expression of proteinases that catabolize the extracellular matrix. Atheromata of *Apoe*^{-/-} – CD4dnTβRII mice had increased levels of matrix metalloproteinase-13 and cathepsin S mRNAs and of the active form of cathepsin S protein but no increase was detected in collagen fragmentation. Our results suggest that exaggerated T-cell-driven inflammation limits collagen maturation in the atherosclerotic plaque while having

little effect on collagen degradation. (*Am J Pathol* 2009, 174:693–700; DOI: 10.2353/ajpath.2009.080561)

A physical disruption of atherosclerotic plaques causes many acute thrombotic complications such as myocardial infarction and stroke.^{1,2} Pathologists have identified a number of characteristics of vulnerable atherosclerotic plaques that have ruptured³ including large lipid cores and thin fibrous caps harboring activated macrophages and T cells.^{1,4} The resistance of the atherosclerotic plaque to disruption depends in part on the integrity of its fibrous cap, which prevents contact between the highly thrombogenic lipid core and the circulating blood.^{1,2} The fibrous cap is composed of smooth muscle cells (SMCs) and a collagen-rich extracellular matrix. The fibrillar collagens types I and III synthesized by SMCs primarily determine the tensile strength of the cap. Sites of plaque rupture characteristically display signs of inflammatory cell activation accompanied by dissolution of matrix.

Inflammation may impair plaque stability because macrophages and mast cells release a set of collagen-degrading matrix metalloproteinases (MMPs) and cysteine proteases.^{5–7} Additional possible mechanisms include inhibited expression of procollagen genes and death or reduced renewal of the collagen-producing SMC population, both phenomena promoted by T-cell-derived interferon (IFN)-γ.⁸ Several studies suggest that T cells may modulate plaque stability. Activated T cells accumulate in vulnerable plaques.⁴ Th1 cells, which dominate in plaques, secrete IFN-γ, a powerful inhibitor of endothelial and SMC proliferation.⁹ IFN-γ also inhibits

Supported by the Leducq Network of Excellence in Atherothrombosis, the Swedish Heart-Lung Foundation (bursary to O.O.), the Swedish Medical Research Council (project grants 6816 and 12660 to G.K.H.), the Academy of Finland (grants 202469 and 1114344 to J.M.), the S. Jusélius Foundation (to J.M.), and the Swedish Institute (Visby Programme for collaboration between the Baltic nations fellowship to O.O.).

Accepted for publication October 22, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

Address reprint requests to Dr. Göran K. Hansson, CMM L8:03, Karolinska University Hospital, SE-17176 Stockholm, Sweden. E-mail: goran.hansson@ki.se.

differentiation and collagen gene expression in SMCs^{10,11} and modulates expression of several MMPs and cathepsins.^{12,13} *In vivo* treatment with IFN- γ increases atherosclerosis and transplant arteriosclerosis,^{14,15} whereas targeted gene deletion in the IFN- γ receptor leads to reduced atherosclerosis in hypercholesterolemic mice.¹⁶ Mice with defective control of T-cell activity show modulation of atherogenesis: interleukin-10 targeted mice display increased plaque formation with reduced collagen accumulation,¹⁷ and *Apoe*^{-/-} mice lacking transforming growth factor (TGF)- β inhibition of T cells rapidly develop large, hyperinflammatory lesions.¹⁸ These data point to a proatherosclerotic and possibly destabilizing role for activated T cells.

The present study aimed to assess the effect of inflammation on the fibrous component of the atherosclerotic plaque. We used *Apoe*^{-/-} $\times$ CD4dnT β RII mice, which lack functional TGF- β receptors on T cells. Therefore, uncontrolled T-cell activation leads to rampant inflammation and accelerated atherosclerosis in hypercholesterolemic animals.¹⁸ Therefore, *Apoe*^{-/-} $\times$ CD4dnT β RII mice provide an opportunity to study the effect of inflammation on atherosclerotic lesions. Our data show reduced enzyme-dependent collagen maturation in hyperinflamed lesions, whereas effects on procollagen expression and collagenolytic enzymes were modest. These results suggest a novel mechanism by which adaptive immunity can modulate plaque stability—impairment of collagen maturation by T-cell-dependent inflammation.

Materials and Methods

Animals

Apoe^{-/-} $\times$ CD4dnT β RII mice were *Apoe*-deficient mice carrying the dominant-negative TGF- β receptor II under the CD4 promoter lacking the CD8 silencer.¹⁸ Female *Apoe*^{-/-} $\times$ CD4dnT β RII transgenic and *Apoe*^{-/-} mice, both on C57BL/6J background, were obtained from in-house breeding at Karolinska University Hospital ($n = 6$ to 9 per group). All mice were fed standard mouse chow and sacrificed under anesthesia at the age of 12 weeks for *Apoe*^{-/-} $\times$ CD4dnT β RII and 12 and 18 weeks for *Apoe*^{-/-} mice to obtain age- and lesion size-matched groups, respectively.¹⁸ All studies were approved by the regional ethical committee for animal experiments.

Tissue Preparation

The aorta was cut between the heart and innominate artery. The proximal part was frozen and cryosectioned from the beginning of aortic root. The rest of the aorta down to the trifurcation was used for preparing extracts of RNA or protein (Supplemental Figure S1, see <http://ajp.amjpathol.org>). Nine 10- μ m sections were collected at 100- μ m intervals starting at a 100- μ m distance from the appearance of the aortic valves. Sections were air-dried and fixed with 4% formaldehyde in phosphate-buffered saline or with ice-cold acetone for histological or immu-

nohistochemical analysis, respectively. Three sections at each 100- μ m interval were kept unfixed at -80°C for *in situ* zymographic assay. Lesion size was analyzed after oil red O staining of formaldehyde-fixed sections at 100- μ m intervals and calculated using Leica QWin image analysis software (Leica, Wetzlar, Germany). The whole intima was included in plaque size measurement whereas lipid staining was used for identification of lesions. For comparative analysis individual lesions between 70,000 and 150,000 μm^2 were carefully matched for size between groups as previously described.¹⁸ Sections used for all analyses were selected within plus or minus 100 μm from maximal lesion size.

Histochemical Analysis

Picrosirius red staining was used for the assessment of collagen fibers in the lesions; it also permits identification of thin collagen fibers not detected with trichrome histochemistry used in our previous study.^{19,20} Briefly, formaldehyde-fixed sections of mouse aortic root were stained for 1 hour in saturated picric acid containing 0.1% picrosirius red (Direct Red 80; Fluka, Buchs, Switzerland). All sections were analyzed under linear polarized light at $\times 40$ magnification in a Leica DMRB microscope and images were captured with a Leica DC480 color video camera. Thick mature collagen fibers were orange-red, and thin immature fibers were in green color.^{20,21} The total collagen content of lesions as well as the content of orange/red and green fibers, respectively, were measured using Leica QWin and calculated as the ratio of thresholded chromogen area to lesion area.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total aortic RNA was isolated from 12-week-old *Apoe*^{-/-} $\times$ CD4dnT β RII and 18-week-old *Apoe*^{-/-} mice by using RNeasy (Qiagen, Hilden, Germany) and analyzed by BioAnalyzer (Agilent Technologies, Waldbronn, Germany) capillary electrophoresis. Reverse transcription was performed with Superscript-II (Life Technologies, Rockville, MD) and random hexamers, and cDNA amplified by real-time PCR in an ABI 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA). For amplification we used TaqMan Universal Master Mix (Applied Biosystems) and premanufactured primers and probes sets (Assay-on-Demand, Applied Biosystems) for $\alpha 1(\text{I})$ and $\alpha 1(\text{III})$ procollagen chains, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14, cathepsins S, L, and K, neutrophil elastase, tissue inhibitor of metalloproteinase-1 (TIMP-1), cystatin C, collagen prolyl-4-hydroxylase (C-P4H) $\alpha(\text{I})$, $\alpha(\text{II})$, and $\alpha(\text{III})$ subunits, lysyl oxidase (LOX), TATA box binding protein (TATAbp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Specific mRNA was expressed as arbitrary units obtained by comparing the threshold cycle value of the test sample with that of a standard curve prepared from aortic mRNA from the same mice. All quantification data were normalized to arbitrary units of reference transcripts.

Western Blot

Tissue extracts (25 μ g of total protein per lane) obtained from total frozen mouse aortas were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and blotted to hybridization transfer membrane Hybond-P (Amersham, Little Chalfont, UK). Blots were blocked and incubated with primary antibodies (rabbit polyclonal anti-LOX: Novus Biologicals, Littleton, CO; goat polyclonal anti-cathepsin S: Abcam, Cambridge, UK; goat polyclonal anti-MMP-13: Chemicon, Single Oak, CA; mouse anti- β -actin: Sigma-Aldrich, St. Louis, MO) and appropriate secondary antibodies diluted in 5% defatted dry milk/Tris-buffered saline/0.1% Tween 20. Immunoreactive proteins were visualized by the ECL Western blotting analysis system (Amersham). Quantification of the bands was performed using digital camera Fujifilm LAS-1000 (Fuji, Tokyo, Japan) and densitometry software Fujifilm Image Gauge version 3.46. The final results are given as the arbitrary units of the intensity of the specific bands normalized to intensity of β -actin staining.

Immunofluorescence

Acetone-fixed sections of the ascending aorta were incubated with rabbit anti-MMP-2 (Chemicon), rabbit anti-TIMP-1 (Affinity BioReagents, Golden, CO), goat anti-MMP-9, or goat anti-MMP-8 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies at 4°C overnight followed by incubation with biotinylated goat anti-rabbit IgG or horse anti-goat IgG, respectively (Vector Laboratories, Burlingame, CA). To quench endogenous autofluorescence in the plaques 0.01% Sudan Black in ethanol was used. Sections were then incubated with streptavidin-conjugated Texas Red (Invitrogen, Carlsbad, CA) and visualized under fluorescent light at $\times 40$ magnification in a Leica DMRB microscope (Leica). Images were captured with a Leica DC 480 color video camera using the same exposure time for every sample. Stained areas were measured at $\times 40$ magnification as the ratio of integrated intensity, ie, the sum of intensity in each pixel, over the entire fluorescent area and divided by lesion area using the Leica QWin morphometry program.

Immunohistochemistry

Adjacent sections of the ascending aorta were stained using mouse anti- α -smooth muscle actin antibody directly conjugated to alkaline phosphatase (Sigma-Aldrich). Staining was visualized using the Vector Red substrate kit (Vector Laboratories). Controls included omission of the primary antibody. Staining was registered as stained surface area, because borders between individual cells could not be identified, and expressed as percentage of stained area to total lesion cross-section area.

In Situ Zymography

The procedure for *in situ* zymography was modified from that previously described.²² In short, quenched fluorescein-labeled gelatin (1 mg/ml, DQ gelatin from pig skin; Molecular Probes, Eugene, OR) was mixed (1:1) with agarose melted in buffer (50 mmol/L Tris-HCl, pH 7.4, 10 mmol/L CaCl_2 , and 0.05% Brij 35) and incubated on unfixed sections of aortic root at 37°C for 24 hours. The fluorescent area produced by proteolytic digestion of quenched fluorescein-labeled gelatin was recognized as combined gelatinase activity (MMP-2 + MMP-9). The generation of fluorescent activity was prevented by addition of the global MMP inhibitors, 1,10 phenanthroline (0.4 mmol/L) and ethylenediaminetetraacetic acid (10 mmol/L). Integrated intensity of green fluorescence of combined gelatinolytic activity was measured as above and divided by lesion area using Leica QWin morphometry program.

Gelatin and Casein Gel Zymography

Aortic lysates (12 μ g) were mixed with native loading buffer 1:1 and separated using either 10% zymography gel (Invitrogen) to document cleavage of gelatin by MMP-2 and MMP-9, or using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with casein substrate (3 mg/ml) to document caseinolytic activity. After electrophoresis the proteins in the gel were renatured and developed. The gel was thereafter stained in Coomassie blue and destained until clear. Gelatinolytic or caseinolytic activity was evident as clear bands against a dark blue background. Quantification of the bands was performed using digital camera Fujifilm LAS-1000 and densitometry software Fujifilm Image Gauge version 3.46.

Cleaved Collagen Assay

Protein extracts of mouse aortas were prepared as described.²³ An immunoassay (RatLaps ELISA; Nordic Bioscience, Herlev, Denmark) was used to detect carboxy-terminal telopeptide of type 1 collagen generated by cleavage of native collagen.

C-P4H Activity Assay

C-P4H activity in aortas of $\text{Apoe}^{-/-} \times \text{CD4dnT}\beta\text{RII}$ and $\text{Apoe}^{-/-}$ mice was assayed by a method based on measurement of the formation of 4-hydroxy-[^{14}C]proline in a [^{14}C]proline-labeled procollagen substrate consisting of nonhydroxylated pro- α chains of chick type I procollagen.²⁴ The assay does not discriminate between the activities of the three different C-P4H isoenzymes but measures the total C-P4H activity generated by all isoforms in a given sample. The C-P4H activity values (dpm/ μ g total protein) were determined in four independent assays for each aortic sample. The final results are given as relative (percent) activities in comparison with control mice.

Statistics

The nonparametric Mann-Whitney significance test and one-way analysis of variance followed by the two-sided *t*-test were used to analyze data. Results in figures are shown as mean $\pm$ SEM.

Results

Reduced Collagen Fiber Maturation in Hyperinflamed Lesions of $Apoe^{-/-}$ $\times$ CD4dnT β RII Mice

The fibrous component of atherosclerotic plaques was evaluated in hyperinflamed lesions of $Apoe^{-/-}$ $\times$ CD4dnT β RII mice and size-matched lesions from control $Apoe^{-/-}$ mice with intact TGF- β modulation of T-cell activation. Collagen composition in lesions was analyzed by using picrosirius red dye, which stains loosely packed, thin, immature collagen fibers green and tightly packed, thick, mature fibers orange-red when visualized in polarized light.^{19,20} The majority of collagen fibers were stained green, indicating a predominance of immature fibers in the lesions of $Apoe^{-/-}$ $\times$ CD4dnT β RII mice (Figure 1, A and E). For comparison, size-matched lesions from control $Apoe^{-/-}$ mice analyzed by the same method contained predominantly mature, orange-red fibers (Figure 1, B and E). Although the total content of interstitial collagen did not differ between lesions of these

two strains (Figure 1E), the ratio of mature/immature (orange-red/green) collagen was 0.59 ± 0.12 in lesions of $Apoe^{-/-}$ $\times$ CD4dnT β RII versus 2.74 ± 0.50 for $Apoe^{-/-}$ mice. The SMC content of lesions, assessed by immunostaining for α -smooth muscle actin (SMA)-positive cells, did not differ between groups (Figure 1, C, D, and F). When age-matched, 18-week-old mice were compared, those of the $Apoe^{-/-}$ $\times$ CD4dnT β RII genotype had more advanced, 38% larger lesions than the $Apoe^{-/-}$ single mutants. Despite this, collagen maturation remained hampered in $Apoe^{-/-}$ $\times$ CD4dnT β RII mice (orange-red/green collagen ratio, 1.89 ± 0.42 versus 2.74 ± 0.50 for $Apoe^{-/-}$). These findings suggest that procollagen gene expression, collagen fiber formation, and/or degradation were affected by the inflammatory state in the artery wall.

Procollagen Gene Expression Unaffected by T-Cell-Driven Inflammation in Plaques

Collagen fiber formation is a multistep process. It starts with transcription and translation of the procollagen gene and is followed by a series of intracellular posttranslational modifications that include glycosylation, prolyl and lysyl hydroxylation, formation of intra- and intermolecular crosslinks, and assembly into a triple-helical structure. After secretion, procollagens are processed into collagen by cleavage of terminal peptides and further modified by lysyl oxidation, which leads to covalent crosslinking of triple-helical collagen molecules into collagen fibers.^{25,26}

Figure 1. Structural composition of lesions. **A** and **B**: Representative micrographs of picrosirius red-stained aortic sections showing collagen fiber composition of atherosclerotic lesions in 12-week-old $Apoe^{-/-}$ $\times$ CD4dnT β RII mice with abrogated TGF- β signaling in T cells (**A**) in comparison with size-matched lesions of 18-week-old $Apoe^{-/-}$ mice with intact TGF- β signaling (**B**). Note predominance of immature, green collagen fibers (single arrow) in **A** and of thick, mature fibers appearing in orange-red (double arrow) in **B**. Polarized light microscopy. **C** and **D**: Adjacent sections stained for α -SMA, bright-field microscopy of immunohistochemical staining. **E**: Collagen composition in lesions, image analysis of micrographs. Data show area of orange (mature fibers), green (immature fibers), and total stained area, respectively, divided by total lesion area. **P* < 0.05. **F**: Smooth muscle content of lesions, image analysis of immunohistochemical staining for α -SMA. No significant difference was observed between groups. Original magnifications: $\times 20$ (**A**, **B**); $\times 10$ (**C**, **D**).

Figure 2. Aortic mRNA expression of type I and III procollagens. Quantitative real-time RT-PCR analysis of type I and III procollagen mRNA in aortas of 12-week-old *Apoe*^{-/-} × CD4dnTRII and of 18-week-old *Apoe*^{-/-} mice with comparable lesion size. Data are expressed as arbitrary units normalized to TATA box binding protein (TATAbp) mRNA. **P* < 0.05.

To identify the molecular target of inflammation, we analyzed three key steps in collagen fiber formation: steady-state procollagen mRNA levels, the intracellular modifying enzyme, collagen prolyl-4-hydroxylase (C-P4H), and the extracellular modifier, LOX.

Procollagen gene expression was evaluated by quantitative real-time RT-PCR of total aortic RNA. Surprisingly, mRNA levels of type I and III procollagen chains did not differ between the two groups (Figure 2), despite the dramatic increase in mRNA for the procollagen-inhibiting cytokines IFN- γ ¹⁸ and interleukin-1 β (data not shown). Similar results were obtained when absolute mRNA levels per ng tissue were compared as when relative levels were calculated after normalization to mRNA for the reference transcript.

Reduced Level of the Collagen-Modifying Enzyme, LOX, in Hyperinflamed Lesions

Enzymatic modification is required to form mature collagen fibers. C-P4H plays a major role in intracellular post-translational modification of procollagen chains. We analyzed mRNA for the three isoforms of the catalytic subunit of C-P4H and observed significantly reduced levels of the α (I) subunit in aortic RNA from *Apoe*^{-/-} × CD4dnTRII mice (Figure 3A). However, α (III) mRNA was increased (Figure 3A) and the total C-P4H enzyme activity was elevated in the aortas of *Apoe*^{-/-} × CD4dnTRII mice (Figure 3B). Therefore, changes in C-P4H cannot explain the reduced collagen fiber maturation observed in the lesions.

Extracellular crosslinking of collagen helices catalyzed by LOX is essential for the formation of collagen fibers. The aortas of *Apoe*^{-/-} × CD4dnTRII had significantly lower levels of LOX mRNA compared with those of

Figure 3. Aortic expression of C-P4H mRNA and enzyme activity. **A:** Quantitative real-time RT-PCR analysis of C-P4H α (I), α (II), and α (III) subunit mRNA in aortas of 12-week-old *Apoe*^{-/-} × CD4dnTRII and 18-week-old *Apoe*^{-/-} mice with similar lesion size. Data are expressed as arbitrary units normalized to TATAbp mRNA. **B:** Analysis of C-P4H activity in aortic lysates of *Apoe*^{-/-} × CD4dnTRII mice and *Apoe*^{-/-} mice. Data are shown as relative C-P4H activity in *Apoe*^{-/-} × CD4dnTRII mice versus controls (%). **P* < 0.05.

Apoe^{-/-} mice (Figure 4A). The amount of active LOX protein (32 kDa) was also reduced, as revealed by Western blot analysis of aortic protein extracts, compared with lesion size-matched as well as age-matched controls (Figure 4B). Densitometric analysis of the 32-kDa LOX band yielded 0.16 ± 0.03 arbitrary units for *Apoe*^{-/-} × CD4dnTRII mice, 0.39 ± 0.07 for lesion-size matched *Apoe*^{-/-}, and 0.29 ± 0.04 for age-matched *Apoe*^{-/-} mice (*P* < 0.05 for *Apoe*^{-/-} × CD4dnTRII mice versus both *Apoe*^{-/-} groups; *n* = 6 per group). These observations strongly suggest that LOX-dependent crosslinking during collagen maturation is reduced in the aorta of the hyperinflamed *Apoe*^{-/-} × CD4dnTRII mouse.

Increased Cathepsin S but Modest Effects on MMPs

Collagenolysis, a process promoted by certain proinflammatory cytokines, may contribute to fibrous cap collagen

Figure 4. Aortic expression of LOX mRNA and protein. **A:** Quantitative real-time RT-PCR analysis of LOX mRNA in aortas of 12-week-old *Apoe*^{-/-} × CD4dnTRII and lesion size-matched *Apoe*^{-/-} mice, arbitrary units normalized to TATAbp mRNA. **P* < 0.05 versus control mice. **B:** Western blot analysis of active LOX protein (32 kDa) in aortic extracts of 18- and 12-week-old *Apoe*^{-/-} mice (a18 and a12) and 12-week-old *Apoe*^{-/-} × CD4dnTRII mice (T). Thus, both age-matched and lesion size-matched lesions of *Apoe*^{-/-} mice were compared with those of 12-week-old *Apoe*^{-/-} × CD4dnTRII mice. Representative Western blots with β -actin as loading control.

Figure 5. Aortic mRNA expression of collagen-degrading proteases. Quantitative real-time RT-PCR analysis of MMPs; TIMP-1; cathepsins K, L, and S; neutral elastase; and cystatin C in aortic extracts of 12-week-old *Apoe*^{-/-} × CD4dnTβRII mice. mRNA levels were normalized to those of TATAbp and are presented as fold change compared with 18-week-old *Apoe*^{-/-} mice. **P* < 0.05 versus control mice.

content. Therefore, we examined mRNA expression of several collagenolytic and elastolytic enzymes in the atherosclerotic aorta. Aortic extracts from *Apoe*^{-/-} × CD4dnTβRII mice contained significantly more mRNA encoding MMP-13 and cathepsin S and less MMP-9 and cystatin C mRNA than controls (Figure 5). mRNA for MMP-2, -3, -8, -12 and -14, TIMP-1, cathepsins K and L, and neutrophil elastase remained unchanged (Figure 5).

In accord with the mRNA data, aortic lysates of *Apoe*^{-/-} × CD4dnTβRII mice had increased immunoreactive cathepsin S protein (30 kDa) (Figure 6A). Western blot analysis of MMP-13 protein, on the other hand, showed reduced amounts of the 55-kDa proform as well as the enzymatically active 45-kDa protein (Figure 6B). Instead, aortas of *Apoe*^{-/-} × CD4dnTβRII mice had an increase in the enzymatically inactive 20-kDa fragment (Figure 6B). This finding suggests that although the increase in MMP-13 mRNA leads to increased enzyme protein, enhanced degradation of this enzyme protein counterbalances its augmented expression.

The distribution of MMP-2, -8, and -9 proteins was assessed by immunofluorescent staining of atherosclerotic lesions and did not differ between the two groups of mice (Supplemental Figure S2, see <http://ajp.amjpathol.org>). The lesions of *Apoe*^{-/-} × CD4dnTβRII mice had significantly reduced TIMP-1 immunoreactivity (Supplemental Figure S2, see <http://ajp.amjpathol.org>), despite equal abundance of TIMP-1 mRNA in the two mouse strains (Figure 5). Interestingly, TIMP-1 protein localized primarily in the subendothelial part of lesions in *Apoe*^{-/-} × CD4dnTβRII mice, but deeper in the lesions and in the

Figure 6. Aortic protein levels of cathepsin S and MMP-13. **A:** Western blot for immunoreactive cathepsin S (30 kDa) in aortic extracts of 12-week-old *Apoe*^{-/-} × CD4dnTβRII mice (T) and of lesion size-matched 18-week-old (a18) and age-matched 12-week-old (a12) *Apoe*^{-/-} mice. **B:** Western blot for immunoreactive MMP-13 in aortic extracts. Shown are the 60-kDa proenzyme, 45-kDa active MMP-13, and 20-kDa enzyme fragment. Groups are as in **A**. Representative Western blots with β-actin as loading control.

Figure 7. Functional analysis of collagenolytic enzymes in the aorta. **A** and **B:** Densitometric analysis of gelatinolytic activity of 92-kDa gelatinase (MMP-9) and 72-kDa gelatinase (MMP-2) (**A**); and total caseinolytic activity (**B**) in lysates of aortas of *Apoe*^{-/-} × CD4dnTβRII mice compared with such in size-matched 18-week-old *Apoe*^{-/-} mice. Data are obtained by gel zymography after electrophoretic separation. Both pro-forms and active forms of gelatinases were included in the analysis. **C:** Immunoassay of C-terminal collagen fragments in aortic extracts showing no difference between *Apoe*^{-/-} × CD4dnTβRII mice and lesion size-matched 18-week-old *Apoe*^{-/-} mice.

media of *Apoe*^{-/-} mice (Supplemental Figure S3, see <http://ajp.amjpathol.org>).

The activity of proteolytic enzymes was analyzed by using gelatin and casein gel zymography of native protein lysates from atherosclerotic aortas. Gelatin zymography showed no difference in MMP-2 activity between the two mouse strains (Figure 7A and Supplemental Figure S4a, see <http://ajp.amjpathol.org>). For MMP-9, results varied between animals with no significant increase in mean densitometric values (Figure 7A and Supplemental Figure S4a, see <http://ajp.amjpathol.org>). Analysis of gelatinase activity by *in situ* gelatin zymography in sections of atherosclerotic lesions, reflecting the combined activity of MMP-2 and MMP-9 did not reveal any difference between *Apoe*^{-/-} × CD4dnTβRII and *Apoe*^{-/-} mice (Supplemental Figure S5, a and b; see <http://ajp.amjpathol.org>). The generation of fluorescent activity was totally prevented by the metalloenzyme inhibitors, phenanthroline and ethylenediaminetetraacetic acid (Supplemental Figure S5c, see <http://ajp.amjpathol.org>). Casein zymography showed no difference in the 20-kDa band corresponding to MMP-7 (Figure 7B and Supplemental Figure S4b, see <http://ajp.amjpathol.org>). However, additional proteases in the lysates from *Apoe*^{-/-} × CD4dnTβRII mice did degrade casein. These bands at 55 and 60 kDa probably corresponded to the stromelysins MMP-3 and MMP-10, respectively (Supplemental Figure S4b, see <http://ajp.amjpathol.org>).

Finally, the amount of cleaved collagen was estimated by using an immunochemical assay detecting a carboxy-terminal telopeptide neoepitope released on proteolytic degradation of type I collagen. This analysis did not reveal any differences in the amounts of collagen fragments between aortic extracts of the two mouse strains, implying that the changes observed for some of the collagenolytic enzymes in the aorta of *Apoe*^{-/-} ×

CD4dnT β RII mice did not result in any increased collagen fragmentation (Figure 7C).

Discussion

The present findings provide direct evidence that inflammation leads to a reduction of mature collagen fibers in atherosclerotic plaques. Although the total collagen content did not differ between *Apoe*^{-/-} × CD4dnT β RII and *Apoe*^{-/-} mice, the hyperinflamed lesions contained fewer mature collagen fibers. Such fibers are tightly packed and crosslinked and exhibit much greater tensile strength than immature collagen fibers,²⁵ therefore the reduced maturation likely results in more fragile plaques with a greater propensity for rupture.

The *Apoe*^{-/-} × CD4dnT β RII mouse exhibits dramatically aggravated immune inflammation because of its dysfunctional control of T-cell activation. Its atherosclerotic lesions abound with activated Th1 effector cells and macrophages.¹⁸ The Th1 cytokine, IFN- γ , inhibits expression of procollagen I and III genes on the mRNA level in cultured SMCs.¹¹ Because of this, we had expected a substantial inhibition of procollagen gene expression. Surprisingly, this was not the case. Lesions from *Apoe*^{-/-} × CD4dnT β RII and *Apoe*^{-/-} mice had similar procollagen I and III mRNA levels. The reduction in mature collagen fibers must result from a more distal defect in the collagen maturation pathway.

Nascent procollagen polypeptides undergo a series of posttranslational modifications, which involve hydroxylation and glycosylation of certain amino acids, intramolecular crosslinking, oligomerization into triple-helical structures, secretion, removal of propeptide sequences, aggregation into collagen fibers, and maturation of these fibers by intermolecular crosslinking.²⁵ Several enzymes catalyze these steps, including hydroxylases, glycosyl transferases, terminal peptidases, and oxidases.²⁶ We analyzed two key enzymes controlling collagen maturation, the intracellular C-P4H and extracellular LOX. Both enzymes contribute crucially to collagen fiber formation and defects in their genes can lead to vascular abnormalities.^{27,28} The stability of collagen helices depends on vitamin C, a co-factor for C-P4H and vitamin C deficiency hampers collagen deposition in atherosclerotic lesions.^{29,30}

Apoe^{-/-} × CD4dnT β RII mice exhibited significantly reduced LOX mRNA and protein in their aortas. For C-P4H, the picture was more complex, with differential effects on mRNA for its different subunits and an increase rather than a decrease in enzyme activity. Therefore, the observed reduction in mature collagen fibers is likely caused by reduced LOX-dependent extracellular crosslinking.

LOX catalyzes the formation of aldehydes from lysine and hydroxylysine residues in collagen. Lysine- and hydroxylysine-derived aldehydes form immature Schiff base crosslinks or ketoimine bonds, which are converted to stable histidine adducts or pyridinium or pyrrolic crosslinks that bind three collagen molecules together.²⁵ *Lox* gene deletion results in fetal death because of vas-

cular abnormalities including large aortic aneurysms.²⁸ Interestingly, IFN- γ , a cytokine overexpressed in the aortas of *Apoe*^{-/-} × CD4dnT β RII mice,¹⁸ inhibits *lox* expression.³¹ LOX levels also decrease with exposure to tumor necrosis factor, another proinflammatory cytokine³²; and in hypercholesterolemia³³ and hyperhomocysteinemia,³⁴ two risk factors for atherosclerotic cardiovascular disease. The present data support a novel mechanism by which activated T cells may hamper plaque stability through regulation of LOX-dependent collagen maturation.

Previous studies by us and others have implicated inhibited SMC proliferation^{10,35} and secretion of collagen-degrading proteases^{20,22,36–38} as major targets for inflammation in atherosclerosis. In the present study however, T-cell-driven inflammation did not significantly affect the SMC content of lesions. Effects on MMPs in lesions were also surprisingly modest, the hyperinflamed aortas only overexpressed MMP-13 mRNA of those MMPs tested. On the protein level, we could not detect any increase in active MMP-13 enzyme but rather an accumulation of MMP-13 fragments. This suggests no net increase in MMP-13 activity under these conditions. In support of this conclusion, an immunochemical assay showed no increase in cleaved collagen.

In addition to MMPs, certain cathepsins may contribute to plaque destabilization.³⁸ Cathepsin S was increased on the mRNA and protein levels and may promote collagenolysis in the hyperinflamed lesions but its effects are likely to be modest because no increase was observed in cleaved collagen. Therefore, T-cell-driven inflammation appears to alter the extracellular matrix of the plaque mainly by inhibiting collagen fiber formation. T-cell activation under conditions differing from those in the *Apoe*^{-/-} × CD4dnT β RII mouse might yield a different program of plaque-modifying activity, although lack of TGF- β regulation of T cells leads to secretion of a broad range of cytokines.^{18,39}

Inflammation in the human plaque likely results from innate as well as adaptive immune activity.² The present study with selective manipulation of triggers originating from the adaptive arm of the inflammatory response permitted dissection of the broader range of plaque-modifying activities. For instance, direct activation of macrophages through pattern recognition ligation induces MMP-9 whereas IFN- γ inhibits its expression.^{12,40} Therefore, our data do not rule out an important role for MMPs in plaque destabilization, they merely show that T cells mainly act on collagen maturation rather than its degradation.

To summarize, our data show that excessive T-cell-driven inflammation in atherosclerotic plaques leads to impaired fibrous cap formation because of reduced amounts of mature collagen fibers. This is caused at least in part by reduced expression of LOX needed for crosslinking of collagen fibers in the extracellular matrix. It will be important to determine whether enzymatic pathways involved in collagen fiber formation are hampered also in human atherosclerosis. If so, they could be interesting targets for plaque stabilizing therapy.

Acknowledgments

We thank Drs. Eugene V. Shlyakhto and Natalia A. Gavrisheva for fruitful discussions; Drs. Emmanuel Tupin and Charlotta Hjerpe for help in animal work; Dr. Richard Flavell for providing CD4dn T_BRII mice; and Inger Bodin, Ingrid Törnbergm, and Liisa Äijälä for technical assistance.

References

- Libby P, Aikawa M: Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med* 2002, 8:1257–1262
- Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005, 352:1685–1695
- Falk E, Shah PK, Fuster V: Coronary plaque disruption. *Circulation* 1995, 92:657–671
- van der Wal AC, Becker AE, van der Loos CM, Das PK: Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 1994, 89:36–44
- Henney AM, Wakeley PR, Davies MJ, Foster K, Hembry R, Murphy G, Humphries S: Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proc Natl Acad Sci USA* 1991, 88:8154–8158
- Kovanen PT: Mast cells: multipotent local effector cells in atherothrombosis. *Immunol Rev* 2007, 217:105–122
- Newby AC: Metalloproteinases and vulnerable atherosclerotic plaques. *Trends Cardiovasc Med* 2007, 17:253–258
- Hansson GK, Libby P: The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol* 2006, 6:508–519
- Hansson GK, Holm J, Jonasson L: Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am J Pathol* 1989, 135:169–175
- Hansson GK, Hellstrand M, Rymo L, Rubbia L, Gabbiani G: Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med* 1989, 170:1595–1608
- Amento EP, Ehsani N, Palmer H, Libby P: Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 1991, 11:1223–1230
- Sarén P, Welgus HG, Kovanen PT: TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol* 1996, 157:4159–4165
- Sukhova GK, Shi GP, Simon DI, Chapman HA, Libby P: Expression of the elastolytic cathepsins S and K in human atheroma and regulation of their production in smooth muscle cells. *J Clin Invest* 1998, 102:576–583
- Whitman SC, Ravisankar P, Elam H, Daugherty A: Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E^{-/-} mice. *Am J Pathol* 2000, 157:1819–1824
- Tellides G, Tereb DA, Kirkiles-Smith NC, Kim RW, Wilson JH, Schechner JS, Lorber MI, Pober JS: Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature* 2000, 403:207–211
- Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C: IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest* 1997, 99:2752–2761
- Caligiuri G, Rudling M, Ollivier V, Jacob MP, Michel JB, Hansson GK, Nicoletti A: Interleukin-10 deficiency increases atherosclerosis, thrombosis, and low-density lipoproteins in apolipoprotein E knockout mice. *Mol Med* 2003, 9:10–17
- Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK: Disruption of TGF-beta signaling in T cells accelerates atherosclerosis. *J Clin Invest* 2003, 112:1342–1350
- Junqueira LC, Bignolas G, Brentani RR: Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 1979, 11:447–455
- Deguchi JO, Aikawa E, Libby P, Vachon JR, Inada M, Krane SM, Whittaker P, Aikawa M: Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. *Circulation* 2005, 112:2708–2715
- Luque EH, Angulo E, Montes GS: A histochemical and electron microscopic study on the collagen of nerves in the domestic fowl. *J Anat* 1983, 137:171–176
- Galis ZS, Sukhova GK, Lark MW, Libby P: Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 1994, 94:2493–2503
- Abdul-Hussien H, Soekhoe RG, Weber E, von der Thülen JH, Kleemann R, Mulder A, van Bockel JH, Hanemaaijer R, Lindeman JH: Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases. *Am J Pathol* 2007, 170:809–817
- Kivirikko KI, Myllylä R: Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. *Methods Enzymol* 1982, 82:245–304
- Gelse K, Poschl E, Aigner T: Collagens—structure, function, and biosynthesis. *Adv Drug Deliv Rev* 2003, 55:1531–1546
- Myllyharju J, Kivirikko KI: Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet* 2004, 20:33–43
- Holter T, Pakkanen O, Soininen R, Sormunen R, Nokelainen M, Kivirikko KI, Myllyharju J: Loss of assembly of the main basement membrane collagen, type IV, but not fibril-forming collagens and embryonic death in collagen prolyl 4-hydroxylase I null mice. *J Biol Chem* 2007, 282:2512–2519
- Mäki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, Soininen R: Inactivation of the lysyl oxidase gene *Lox* leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. *Circulation* 2002, 106:2503–2509
- Nakata Y, Maeda N: Vulnerable atherosclerotic plaque morphology in apolipoprotein E-deficient mice unable to make ascorbic acid. *Circulation* 2002, 105:1485–1490
- Libby P, Aikawa M: Vitamin C, collagen, and cracks in the plaque. *Circulation* 2002, 105:1396–1398
- Song YL, Ford JW, Gordon D, Shanley CJ: Regulation of lysyl oxidase by interferon-gamma in rat aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000, 20:982–988
- Rodríguez C, Alcudia JF, Martinez-Gonzalez J, Raposo B, Navarro MA, Badimon L: Lysyl oxidase (LOX) down-regulation by TNFalpha: a new mechanism underlying TNFalpha-induced endothelial dysfunction. *Atherosclerosis* 2008, 196:558–564
- Rodríguez C, Raposo B, Martinez-Gonzalez J, Casani L, Badimon L: Low density lipoproteins downregulate lysyl oxidase in vascular endothelial cells and the arterial wall. *Arterioscler Thromb Vasc Biol* 2002, 22:1409–1414
- Raposo B, Rodríguez C, Martinez-Gonzalez J, Badimon L: High levels of homocysteine inhibit lysyl oxidase (LOX) and downregulate LOX expression in vascular endothelial cells. *Atherosclerosis* 2004, 177:1–8
- Hansson GK, Holm J: Interferon-gamma inhibits arterial stenosis after injury. *Circulation* 1991, 84:1266–1272
- Sukhova GK, Schonbeck U, Rabkin E, Schoen FJ, Poole AR, Billingham RC, Libby P: Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation* 1999, 99:2503–2509
- Sukhova GK, Zhang Y, Pan JH, Wada Y, Yamamoto T, Naito M, Kodama T, Tsimikas S, Witztum JL, Lu ML, Sakara Y, Chin MT, Libby P, Shi GP: Deficiency of cathepsin S reduces atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2003, 111:897–906
- Liu J, Sukhova GK, Sun JS, Xu WH, Libby P, Shi GP: Lysosomal cysteine proteases in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2004, 24:1359–1366
- Gorelik L, Flavell RA: Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 2000, 12:171–181
- Gebbia JA, Coleman JL, Benach JL: Selective induction of matrix metalloproteinases by *Borrelia burgdorferi* via toll-like receptor 2 in monocytes. *J Infect Dis* 2004, 189:113–119