Modulation of the Extracellular Matrix in Advanced Atherosclerosis

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2 Overexpression of Interleukin-18 Decreases Intimal Collagen Content and Promotes a Vulnerable Plaque Phenotype in Apolipoprotein-E Deficient Mice

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Arterioscler Thromb Vasc Biol. 2004 Dec;24(12):2313-9.

Abstract

Although interleukin-18 (IL-18) has been implicated in atherosclerotic lesion development, little is known about its role in advanced atherosclerotic plaques. This study aims to assess the effect of IL-18 overexpression on the stability of pre-existing plaques. Atherosclerotic lesions were elicited in carotid arteries of apoE deficient mice (n=32) by placement of a perivascular collar. Overexpression of IL-18 was effected by i.v. injection of an adenoviral vector five weeks after surgery. Two weeks after transduction, lesions were analyzed histologically with regard to plaque morphology and composition or by real-time PCR. No difference in plaque size was detected between groups. In the Ad.IL-18 treated group 62% of lesions displayed a vulnerable morphology or even intraplaque hemorrhage as compared to only 24% in the controls (P=0.037). In agreement, IL-18 overexpression reduced intimal collagen by 44% (P<0.003) and cap-to-core ratio by 41% (P<0.002). While IL-18 did not affect the expression of collagen synthesis related genes, it was found to enhance the collagenolytic activity of vSMCs in vitro, suggesting that the low collagen content is attributable to matrix degradation rather than to decreased synthesis. Systemic IL-18 overexpression markedly decreases intimal collagen content and plaque thickness leading to a vulnerable plaque morphology.

Introduction

Numerous reports have indicated that inflammatory processes play a pivotal role throughout plaque development as well as in plaque rupture and thrombosis.[1,2] One of the proinflammatory mediators that has received considerable attention in this regard is interleukin-18 (IL-18). It is an IL-1 family member [3], involved in Th1 cell activation and expansion and induces IFN-γ, a known proatherogenic mediator [4,5], in cultured macrophages and in smooth muscle cells, but not in endothelial cells.[6] Ligand binding to the IL-18 receptor results in the enhanced secretion of many other cytokines and proteins causally involved in atherosclerosis among which IL-6, IL-8, ICAM-1 and various matrix metalloproteinases (MMPs).[6] IL-18 and its receptor are expressed in human atheroma-associated endothelial cells, vascular smooth muscle cells (vSMCs) and macrophages, and their expression is enhanced upon stimulation with IL-1β and TNF-α.[6] In mouse models, IL-18 enhanced aortic atherogenesis in apolipoprotein E (apoE) deficient mice through release of IFN-γ.[7] Conversely, IL-18 deficiency and IL-18 Binding Protein (IL-18BP) attenuated lesion development and progression and it was suggested to promote plaque stability during initial lesion formation.[8,9]

Although the role of IL-18 in atherosclerotic lesion formation is well established, its effect at later stages of plaque development on plaque stability is less well investigated. Epidemiological studies in humans pointed to a destabilizing role for IL-18 in more advanced stages of plaque development. Indeed, IL-18 serum levels have been found to correlate to cardiovascular morbidity and mortality in patients with coronary heart disease.[10,11,12,13] In addition, Mallat and colleagues reported elevated IL-18 mRNA levels in unstable human plaques from carotid endarterectomy.[14]

Plaque rupture is the predominant cause of acute thrombosis leading to vascular occlusion and ischemia. The fibrous cap maintains the structural integrity of the atheromatous lesion. A dysbalance in synthesis and degradation of the extracellular matrix leads to thinning of the cap and renders the plaque more prone to rupture. It is conceivable that IL-18 promotes this dysbalance through the induction of IFN-γ, apoptosis or protease activity. Various matrix metalloproteinases (MMPs) and cathepsins are overexpressed in vulnerable plaques and induced by pro-inflammatory mediators.[15,16,17,18] In addition, inflammatory processes can reduce the number of intimal cells or their ability for collagen synthesis, thereby compromising matrix production and threatening plaque stability.

Since little is known about the effects of IL-18 on the composition and stability of advanced atherosclerotic plaques, we aimed to assess the effect of systemic IL-18 overexpression on the morphology and stability of pre-existing complex lesions in apoE deficient mice. To this end, we injected an IL-18 expressing adenovirus to increase circulating IL-18 in mice with advanced, collar-induced lesions in the common carotid artery.[19] IL-18 overexpression significantly decreased intimal collagen content, which was accompanied by a more rupture prone plaque morphology, indicating that IL-18 overexpression decreases atherosclerotic plaque stability.

Materials and Methods

Animals
Female apoE deficient mice (n=32), 10-12 weeks of age, were obtained from our own breeding stock. Mice were placed on a western-type diet containing 0.25% cholesterol (Special Diets Services, Witham, Essex, UK). High fat diet and water were provided ad libitum. All animal work was approved by the regulatory authority of Leiden University and performed in compliance with the Dutch government guidelines.

Carotid collar placement and transgene expression
Carotid atherosclerotic lesions were induced by perivascular collar placement as previously described.[19] Mice were anesthetized with ketamine (60 mg/kg; Eurovet, Bladel, Netherlands), fentanyl citrate and fluanisone (1.26 mg/kg and 2 mg/kg, respectively; Janssen Animal Health). Five weeks after surgery the animals were injected intravenously with 200 µl of a suspension of adenovirus ($5.0 \cdot 10^9$ pfu/mL) carrying a murine IL-18 or an empty transgene under control of a CMV promoter (Ad.IL-18 and Ad.Empty respectively).[20] Two weeks later, lesions from both carotids were analyzed histologically (n=16-17) with regard to plaque morphology and composition or by real time PCR (n=14).

Plasma analysis
Weekly blood samples were taken and plasma cholesterol levels were monitored using enzymatic procedures (Roche Diagnostics). Triglyceride levels were quantified using a commercially available kit (Roche). Precipath standardized serum (Boehringer Mannheim) was used as an internal standard. Murine IL-18 levels were determined by ELISA (OptEIA™ Set Mouse IL-18, BD Biosciences, San Diego, CA) one week after injection of the adenoviral vector according to the manufacturer's instructions.

Tissue harvesting and preparation for histological analysis
Mice were sacrificed two weeks after infection. One day prior, phenylephrine (8 µg/kg i.v.; Sigma Diagnostics, St. Louis, MO) was administered to all animals in order to asses the effect on plaque integrity via a hemodynamic challenge. Serial cryosections (5µm thick) were prepared from carotid artery and stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica). Collagen staining was performed by a 90 minute incubation in 0.1% Sirius Red (Direct red 80, Sigma) in saturated picric acid. Corresponding sections were stained immunohistochemically with antibodies directed against mouse metallophilic macrophages (monoclonal mouse IgG_{2a}, clone MOMA2, dilution 1:50; Sigma Diagnostics, St. Louis, MO) and against α-SM-actin (monoclonal mouse IgG_{2a}, clone 1A4, dilution 1:500; Sigma Diagnostics, St. Louis, MO). To assess intimal cell death, sections were subjected to TUNEL staining using protocols provided by the manufacturer (In Situ Cell Detection Kit, Roche Diagnostics).

Tissue harvesting and preparation for expressional analysis
For expression analysis, freshly isolated non-fixed plaques were pooled in three groups per treatment group for RNA isolation using the TriZol method (Invitrogen, Breda, Netherlands). Purified RNA was DNase treated (DNase I, 10U/µg total RNA) and reverse transcribed (RevertAid M-MulV Reverse Transcriptase) according to the protocols provided by the manufacturer. Quantitative gene expression analysis was performed on an ABI PRISM 7700 machine (Applied Biosystems, Foster City, CA) using SYBR Green technology. Primers were designed for murine α-SM-actin, Matrix Gla Protein (MGP), desmin, CD68, IL-1β, MMP-3, -9 and -13, heat shock protein 47 (hsp47), procollagen type I α_2 and IL-18 using PrimerExpress 1.7 software (Applied Biosystems) and validated for identical efficiencies (table 1). Target gene mRNA levels were expressed relative to the housekeeping gene (36b4) and calculated by subtracting the threshold cycle number (Ct) of the target gene from the Ct of 36b4 and raising two to the power of this difference.

Morphometry
The site of maximal plaque size was selected for morphometric assessment and images were digitized and analyzed as previously described [19]. In addition, cap and core areas were measured as well as mean cap thickness. Stage of lesion progression was assessed according to the classification criteria defined by Virmani et al..[21] Out of six categories (i.e 1: fibrous lesion, 2: atheromatous lesion, 3: thin cap atheroma, 4: healed rupture, 5: plaque rupture or intraplaque hemorrhage and 6: plaque erosion) the first two classes are considered stable whereas lesions in classes 3 to 6 are perceived as plaques with characteristics of vulnerability. Lesions were blindly allocated to the different classes using observations of subsequent sections with a maximal interval of 100 µm. Thin cap atheroma were defined as plaques with fibrous caps with an average thickness of ≤3 cell layers. Occlusive thrombus formation without signs of plaque rupture or bleeding was categorized as plaque erosion.[22] Macrophage- and collagen-positive areas were determined by computer-assisted color-gated measurement, and related to the total intimal surface area. TUNEL positive cells were counted and related to the total number of intimal cells.

Table 1. Taqman primersets

Gene	Forward primer	Reverse primer
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
α-SM-actin	TCCCTGGAGAAGAGCTACGAACT	GATGCCCGCTGACTCCAT
Desmin	GATGCAGCCACTCTAGCTCGTATT	CTCCTCTTCATGCACTTTCTTAAGG
Hsp47	ACAAGATGCGAGATGAGTTGTAGAGT	TAGCACCCATGTGTCTCAGGAA
MGP	GCATGTGTTGCTTGCTCCTTAC	TCATTACTTTCAACCCGCAGAA
MMP-3	TTTAAAGGAAATCAGTTCTGGGCTATAC	CGTAAGTGTGGGACCCAGAC
MMP-9	CTGGCGTGTGAGTTTCCAAAAT	TGCACGGTTGAAGCAAAGAA
MMP-13	CAACCTATTCCTGGTTGCTGC	ATCAGAGCTTCAGCCTTGGC
Osteopontin	CAGGCATTCTCGGAGGAAC	GAGCTGGCCAGAATCAGTCACTTT
Procol I	TGTACTATGGATGCCATCAAAGTGT	CCATTGATAGTCTCTCCTAACCAGACA
Procol III	TGCCCAACTGCGCTTCA	CCAGCCTGACAGGTTGGAAA
TIMP-1	ACACCCCAGTCATGGAAAGC	CTTAGGCGGCCCGTGAT

Detection of protease activity by gelatin and collagen zymography
Murine hepatoma cells (mhAT3F2 from mice transgene for SV40 driven by an antithrombin III promoter)[23] were plated 24h before infection at the required cell density. Ad.IL-18 or Ad.Empty was added to the cells at 500 MOI in 500 µl of fresh media and left for 16h at 37°C. Cells were washed and incubated in fresh media for 24h before collection of the conditioned media. Samples were centrifuged, added to murine vascular smooth muscle cells (vSMCs) or RAW 264.7 cells and left for 24h at 37°C. Primary vSMCs were isolated from C57Bl/6 murine aortas as previously described.[24]
To investigate gelatinase and collagenase activity after incubation with the conditioned media, samples were subjected to gelatin and collagen zymography. Briefly, the conditioned media samples were centrifuged to dispose of cellular debris, kept on ice and processed immediately after incubation. Equal volumes of media (25 µL) were added to 25 µL sample buffer (0.125M Tris-HCl, 20% glycerol, 4% SDS, 0.005% Bromophenol Blue; pH 6.8). Equal volumes of the samples were loaded onto a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and 1.5 mg/ml gelatin or 1 mg/ml collagen. Following electrophoresis gels were incubated in 2.5% Triton-X-100 for 30 min, in developing buffer (0.05 M Tris, 0.05 M NaCl, 0.01 M $CaCl_2$ and 0.02% Brij-35) for 16h at 37°C and subsequently stained with 0.5% Coomassie Brilliant Blue for 30min. After destaining, bands of lysis representing protease activity were visualized as stainless spots against a blue background.

Statistics
Differences in plaque size were statistically analyzed for significance using the Mann-Whitney U test. Other plaque parameters, collagen and macrophage content, as well as TUNEL staining and differences in ΔCt were compared using the two-tailed Student's *t*-test. Correlations were determined with Spearman's rank correlation test. Differences in the occurrence of adverse events and in classification were analyzed with the Yate's corrected two-sided Fisher's exact test.

Results

Throughout the experiments the mice remained in good health and adenoviral gene transfer was tolerated very well. Neither bodyweight nor plasma cholesterol levels were affected by any of the procedures. IL-18 plasma levels were measured one week post infection. Intravenous administration of $1 \cdot 10^9$ pfu's of Ad.mIL-18 led to a nine-fold increase of circulating IL-18, which was raised from 150±200 pg/mL to 1350±650 pg/mL (figure 1).

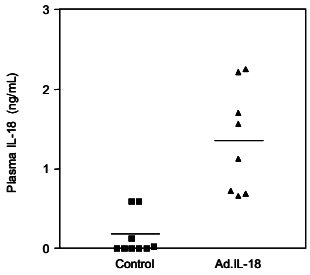

Figure 1. Adenoviral vectors target to the liver, which then secretes the gene product into the circulation. One week after administration of Ad.IL-18 plasma levels of IL-18 (pg/mL) increased nine-fold compared to those in the control group (P=0.0001).

IL-18 overexpression did not affect size of advanced plaques

Since the aim of this study was to assess the effect of IL-18 on pre-existing, advanced atherosclerotic plaques, it was important to induce lesions at a predefined place in a time-controlled fashion prior to administration of the adenoviral vectors. In this way, neither plaque size nor location of the lesion could be held accountable for a change in plaque composition or stability. For this reason we applied our collar model for rapid atherogenesis, in which the placement of a perivascular collar on the common carotid artery induces atherosclerotic plaques proximal to the collar within 4 to 6 weeks. Five weeks after surgery, the animals were injected intravenously with the adenoviral vectors and two weeks later the carotid arteries were harvested for further analysis.
No difference in plaque size was detected between groups (Ad.IL-18: 49,000±5,000 μm^2 vs. Ad.Empty: 53,000±5,000 μm^2), neither did we find any difference in intima/lumen ratios (figure 2 A & D). Media size and intima/media ratios did not differ between groups (figure 2 B & C) suggesting that the degree of outward remodeling was not affected by IL-18.

Systemic IL-18 overexpression led to vulnerable plaque morphology

The main objective of this study was to assess the effect of IL-18 on plaque stability. For this, lesions were categorized according to their morphological features. We opted to apply the classification as described by Virmani and colleages.[21] Fibrous lesions and atheromatous plaques, class 1 and 2, were perceived as stable. Plaques showing thin cap morphology, defined as having a cap thickness≤ 3 cell layers, or adverse events, like intraplaque hemorrhage or intramural thrombosis, (classes 3 to 6) were considered unstable. In the Ad.IL-18 treated group 62% of the lesions displayed features of a vulnerable plaque morphology as compared to only 24% in the controls (P=0.037) (table figure 3). Adverse events (class 5), in this case intralesional bleedings (an established signs of plaque vulnerability), were observed in 19% of IL-18 overexpressing but not in control plaques (figure 3). Plaque rupture or erosion could not be detected. To confirm the higher incidence of thin cap atheroma, fibrous cap thickness was measured at twelve different, evenly spaced sites of the cap. Mean cap thickness decreased 41% after IL-18 exposure (Ad.Empty: 17.0±1.5 µm vs. Ad.IL-18: 9.9±3.0 µm; P=0.026) (figure 2E). Likewise, cap area (Ad.Empty: 7,720±480 μm^2 vs. Ad.IL-18: 4,190±750 μm^2; P=0.0002) and cap:core ratio (figure 2F) (Ad.Empty: 0.17±0.01 vs. Ad.IL-18: 0.10±0.02; P=0.002) decreased significantly.

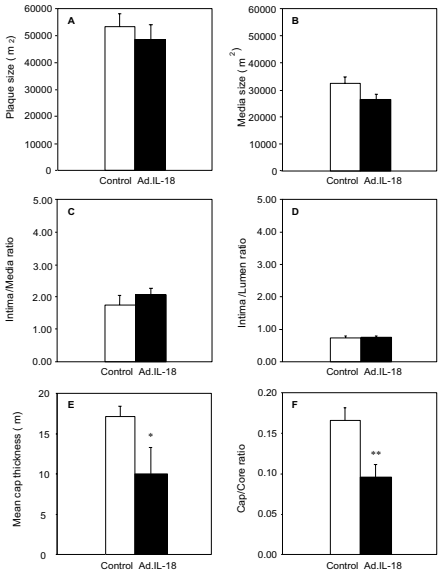

Figure 2. Baseline characteristics of the plaques were comparable between groups. Two weeks after transduction, the Ad.Empty and Ad.IL-18 treated mice did not show any differences in morphometric parameters such as plaque size (A), media size (B), intima/media ratio (C) and intima/lumen ratio (D). Mean cap thickness as measured at twelve different points per section differed significantly (E) as did the cap:core ratio (F). * P=0.026, ** P=0.002. Values are mean ± SEM.

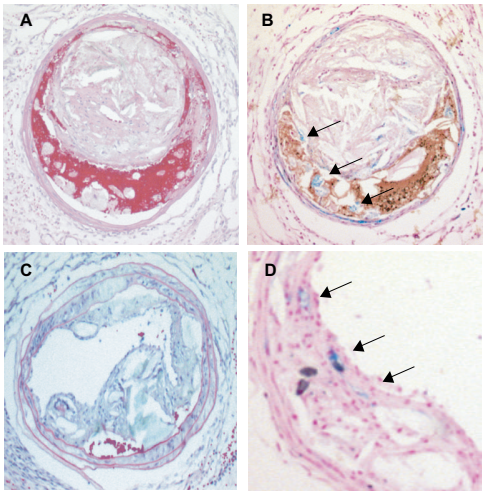

Figure 3. Massive intraplaque hemorrhage on HE staining (A+C) and Perl's staining for iron (B+D). Arrows show deposition of iron, the product of degraded hemoglobin, suggestive for the presence of intramural thrombi.

The table displays the distribution of collar induced carotid lesions showing stable or vulnerable plaque morphology. The IL-18 treated group showed a higher prevalence of unstable plaques (62% vs. 24% in the control animals, P=0.037).

	Control	**IL-18 overexpression**
Stable	13 (76%)	6 (38%)
Fibrous plaque	7	1
Atheromatous plaque	6	5
Unstable	4 (24%)	10 (62%)
Thin cap atheroma	4	7
Intraplaque hemorrhage	0	3

Effects on plaque composition

The IL-18 group showed a 44% decrease in intimal collagen content (collagen/intima ratio 0.22 vs. 0.39, P=0.003) (figure 4 A-C). Since lesions had already progressed to an advanced stage in both groups it is unlikely that the observed depletion of collagen already existed at the time of introduction of IL-18. Low collagen content was mainly observed in plaques considered unstable and thus largely class-dependent. Intimal collagen significantly correlated to the morphological class (R=-0.79, P<0.0001) (figure 4D). Still, although not reaching statistical significance, within the same morphological classes a slight reduction in intimal collagen in the Ad.IL-18 treated group could be noticed (data not shown). Furthermore, IL-18 plasma levels were negatively correlated with intimal collagen content (R=-0.45, P=0.01), suggesting that there is an intrinsic effect of IL-18 on collagen homeostasis (data not shown).

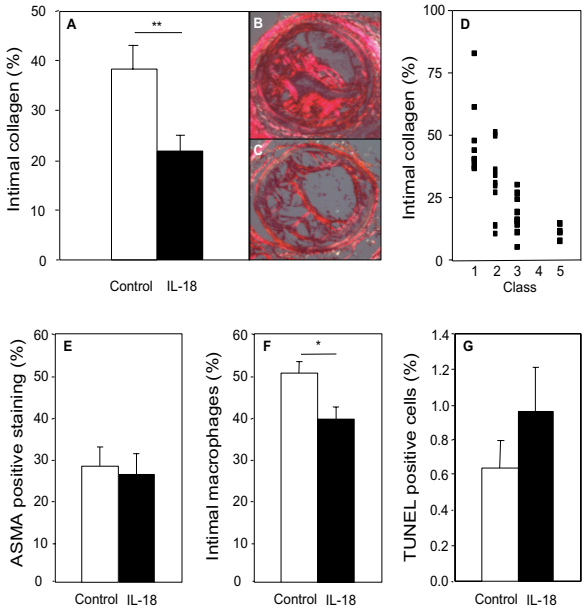

Figure 4. A. Intimal collagen (%). 40% decrease in IL-18 treatment group. **B+C.** Picro Sirius red stained sections of lesions from control animals (B) and Ad.IL-18 treated mice (C). **D.** Correlation between intimal collagen and morphological class (R=-0.79, P<0.0001). **E.** Intimal smooth muscle cells. Percentage ASMA positive staining in the intimal. **F.** Percentage intimal macrophages. **G.** Percentage TUNEL positive cells. Values are mean ± SEM. * P=0.009 , ** P=0.003.

Cell density may reflect the capacity of the intima to produce matrix components. For this reason, we quantified the number of cells relative to the intimal area, showing that IL-18 treatment moderately decreased intimal cell density (0.0044±0.0013 vs. 0.0057±0.0014 nuclei/μm^2 in the controls; P=0.044) (data not shown). However, relative smooth muscle cell content as determined with α-actin staining did not differ between groups (Ad.IL-18 treated: 26% vs. Ad.Empty treated: 28%; P=0.76) (figure 4E). Interestingly, a decrease in the relative macrophage content of the lesion was observed in Ad.IL-18 treated mice (39% vs. 51%, P=0.009), which was accompanied by an increase of the relative amount of necrotic core area at the site of maximal plaque size (figure 4F). The latter also might explain the reduction in intimal cell density. Finally, the rate of apoptosis was assessed by means of a TUNEL staining.

Although an increase in the amount of TUNEL positive cells could be observed this did not reach statistical significance (P=0.28) (figure 4G).

Effects on gene expression within the lesion

To elucidate the mechanism of the observed decrease of intimal collagen we performed real-time PCR analysis on lesional mRNA. This neither revealed an altered gene expression of markers for the synthetic phenotype of vSMCs, like Matrix Gla Protein (MGP), nor of those for the contractile phenotype (e.g. desmin). Also, a difference in the expression of the α2 chain of procollagen type I, the major collagen constituent of the plaque, and the chaperone hsp47, involved in collagen processing [25,26], could not be detected (figure 5). These data suggest that the rate of collagen synthesis remained unaltered after exposure to IL-18 and that processes other than phenotypic modulation or collagen production might be responsible for underlying the observed decrease in intimal collagen content.

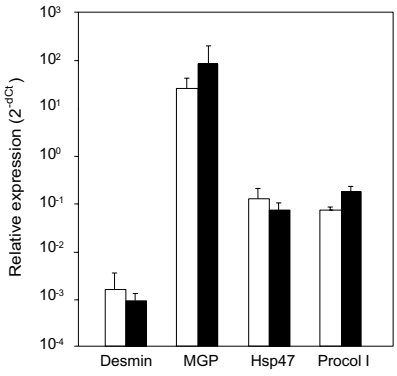

Figure 5. Intralesional expression levels of several key genes for smooth muscle cell phenotype and for collagen synthesis. None of these genes were significantly up- or downregulated after introduction of IL-18. Values are mean ± SD.

IL-18 causes increased proteolytic activity of vascular smooth muscle cells in vitro

Since procollagen type I expression did not decrease and smooth muscle cell number and phenotype did not seem to change upon IL-18 treatment, it is plausible that the observed decrease in collagen content may be attributable to increased matrix degradation rather than to an impaired synthesis.
The effect of IL-18 on the proteolytic capacity of vascular wall cells was tested in vitro. Murine hepatoma cells were infected with Ad.IL-18 or with mock virus, conditioned media were collected 24h after removal of the virus and murine vSMCs and RAW 264.7 cells were incubated with this media for 24h. RAW 264.7 cells were used to exclude any paracrine effects (e.g. IFN-γ induction) that may result from even a minor lymphoid contamination.[27] Zymographic analysis of the culture media showed that gelatinolytic and collagenolytic activity in the RAW 264.7 supernatant remained unaffected (data not shown). By contrast, vSMCs displayed an increased

collagenolytic activity at 53 kDa corresponding to MMP-13 (40.0±5.1 INT·mm^2 in the controls vs. 108.0±9.1 INT·mm^2 in the Ad.IL-18 treated group, P=0.0003) (figure 6A). No effect could be seen on the activity of the gelatinases MMP-2 (figure 6B) and –9 (not shown).
In line with the *in vivo* observations, expression levels of genes indicating vSMC phenotype and genes involved in collagen synthesis were unaffected (figure 6C). Also, MMP mRNA levels remained unchanged upon IL-18 treatment (figure 6D) suggesting that IL-18 did not alter the expression of MMP-13, but rather its secretion or activation.

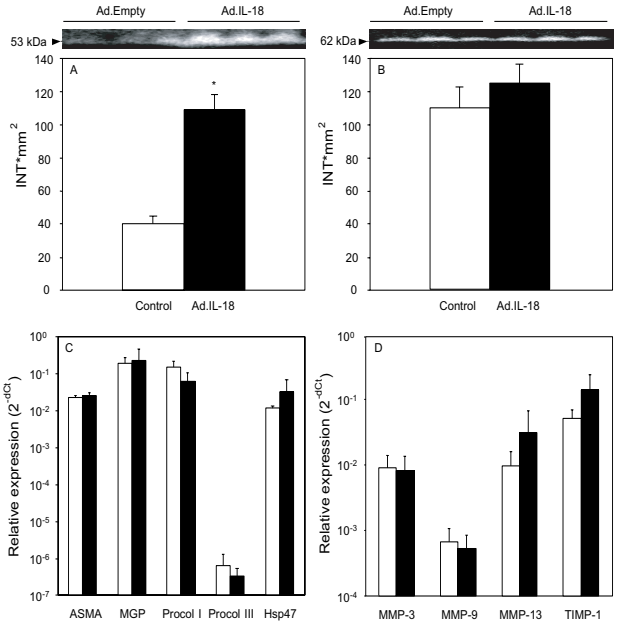

Figure 6. A. Collagen zymography. Conditioned media from Ad.IL-18 transduced hepatoma cells caused an increase in MMP-13 activity in VSMCs. * P=0.0003. **B.** Gelatin zymography. No change could be detected in MMP-2 and –9 activity. Values are mean ± SEM. **C+D.** Gene expression in vSMCs after incubation with media of Ad.Empty or Ad.IL-18 transduced hepatocytes. Collagen synthesis (C) and MMP expression (D) are unchanged after IL-18 treatment. Values are mean ± SD.

Discussion

The importance of IL-18 in atherosclerosis is well established. Its proatherogenic effect, at least in male apoE deficient mice, appears to be mediated by IFN-γ, both in a T-cell dependent and independent fashion.[7] In our study, we used female mice. Intriguingly, IFN-γ deficiency did not attenuate atherogenesis in females [28] to a similar extent as it did in males and therefore it seems less likely that the observed plaque destabilising activity of IL-18 involves an IFN-γ dependent process. Although several epidemiological reports suggest an involvement of IL-18 in plaque rupture and acute coronary events [9,10,11,14,29,30], studies addressing its role in plaque stability are surprisingly lacking and no direct evidence of causality has been shown to this date. Our observations, for the first time, causally link IL-18 to plaque destabilization. Furthermore, our results show, that IL-18 influences matrix biology by modulating MMP activity in vSMCs.

In our model for rapid atherogenesis, introduction of an adenoviral vector carrying a murine IL-18 transgene caused an elevation of circulating IL-18. Since the size of the pre-existing lesions was not affected by IL-18 transduction, the observed changes in plaque composition and morphology could not be explained by a difference in plaque size. Systemic overexpression of IL-18 caused a marked decrease in intimal collagen and led to a plaque phenotype with clear characteristics of vulnerability, i.e. thin cap morphology with large necrotic cores. In three of the IL-18 treated vessels intraplaque hemorrhage was observed, accompanied with iron deposits, reflective of an intramural thrombus. Neither collagen type I expression, nor the amount of vSMCs and the incidence of apoptosis was significantly changed after introduction of IL-18. In vitro, an increase of MMP-13 activity was detected in vSMCs, but not in macrophages. This suggests that intimal collagen had been diminished by degradation rather than by reduction of its synthesis. The surprising decrease in relative macrophage content might be a reflection of a further progressed plaque phenotype, accompanied by less collagen, larger necrotic cores and a higher prevalence of thin cap atheroma after IL-18 overexpression.

For structural integrity the atheroma relies on collagen type I and, to a lesser extent, type III [31], the homeostasis of which is maintained at various levels. Collagen synthesis within the plaque is mainly attributable to vSMCs [32] and modulated by various growth factors and cytokines, either directly, by influencing procollagen expression, or indirectly, by shifting the VSMC phenotype from a contractile to a synthetic state. IL-18 could indirectly affect collagen production by inducing IFN-γ, which in turn has been shown to inhibit IL-1, TGF-β and PDGF induced collagen synthesis.[33]

In our study, procollagen type I mRNA levels did not decrease at the site of the lesion and the expression of desmin and MGP remained constant. Therefore it is unlikely that collagen synthesis was diminished, either by transcriptional downregulation via IFN-γ stimulation or by phenotypic modulation. Also, the expression of hsp47, an important chaperone in intracellular trafficking and processing of procollagen, did not change within the plaque.

In addition to regulating matrix production directly, inflammatory mediators could affect collagen deposition indirectly by promoting apoptosis of collagen-synthesizing cells.[34,35,36] Earlier observations in our lab indicate that induction of vSMC apoptosis in the fibrous cap will decrease cell density resulting in cap thinning and therewith plaque destabilization.[37] Recently, IL-18 has been shown to promote apoptosis by stimulating the secretion of FasL and the expression of TNFR-I on the cell surface.[38,39] Although in this study a slight increase in apoptosis could be noticed in the IL-18 treatment group, it did not reach a level of significance. Furthermore, α-SM-actin staining showed a very moderate and insignificant decrease of intimal vSMCs after IL-18 overexpression. Therefore, loss of collagen synthesizing cells alone cannot fully explain our observations.

Besides collagen production, structural integrity is dependent on proteolytic activity. vSMCs as well as macrophages secrete a wide array of different proteases and protease inhibitors. In particular, matrix metalloproteinases have been associated with both atherogenesis and plaque destabilization.[15,16,17,40,41] Gerdes et al. reported a stimulatory effect of IL-18 on MMP-1, -9 and –13 protein expression in human macrophages [6], while targeted deletion of caspase-1 not only decreased IL-18 production, but also that of MMP-3.[42] Each of these proteases was shown to be highly expressed in unstable atherosclerotic plaques.[17,18]

In our study, we tested the effect of IL-18 exposure on vSMCs and macrophages. While even a minor contamination with lymphoid cells can result in a

paracrine activation of primary macrophages,[27] we opted to use RAW 264.7 cells. IL-18 had no effect on MMP expression or gelatinolytic activity in vitro and was found only to enhance MMP-13 activity of vSMCs but not of RAW 264.7 macrophages, albeit that we cannot exclude that MMP activity of macrophages in plaques is not responsive to IL-18 as well. Although, macrophages are considered to be the main source of MMPs in atherosclerosis [43], this study showed that vSMCs can also play their part in cap thinning and plaque destabilization. Since vSMC MMP-13 expression was not affected it seems that IL-18 enhanced its secretion or activation rather than its production. These findings raise the possibility that matrix degradation through MMP activation is a major culprit in IL-18 induced collagen reduction.

In summary, systemic IL-18 overexpression caused a marked decrease in intimal collagen content and led to vulnerable plaque morphology in apoE deficient mice. The elevated MMP-13 activity in vitro suggests that excessive matrix degradation could be responsible for the observed shift towards a vulnerable plaque morphology. This proteolytic activity may be executed by vSMCs, which may attenuate the importance of macrophage infiltration as a conditio sine qua non for thinning of the fibrous cap. In conclusion these data underline the importance of IL-18 in managing extracellular matrix integrity and in plaque stability, making it an attractive target for therapeutic intervention.

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