Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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

R. de Nooijer, J.H. von der Thüsen, C.J.N. Verkleij, J. Kuiper, J.W. Jukema, E.E. van der Wall, Th.J.C. van Berkel and E.A.L. Biessen

Arterioscler Thromb Vasc Biol. 2004;24:2313-2319; originally published online October 7, 2004;
doi: 10.1161/01.ATV.0000147126.99529.0a
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636

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

R. de Nooijer, J.H. von der Thüsen, C.J.N. Verkleij, J. Kuiper, J.W. Jukema, E.E. van der Wall, Th.J.C. van Berkel, E.A.L. Biessen

Objective—Although 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 preexisting plaques.

Methods and Results—Atherosclerotic lesions were elicited in carotid arteries of apolipoprotein E (apoE)-deficient mice (n=32) by placement of a perivascular collar. Overexpression of IL-18 was effected by intravenous injection of an adenoviral vector 5 weeks after surgery. Two weeks after transduction, lesions were analyzed histologically with regard to plaque morphology and composition or by real-time polymerase chain reaction. 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 with 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% ($P<0.002$). Although IL-18 did not affect the expression of collagen synthesis-related genes, it was found to enhance the collagenolytic activity of vascular smooth muscle cells in vitro, suggesting that the low collagen content is attributable to matrix degradation rather than to decreased synthesis.

Conclusion—Systemic IL-18 overexpression markedly decreases intimal collagen content and cap thickness, leading to a vulnerable plaque morphology. (***Arterioscler Thromb Vasc Biol.* 2004;24:2313-2319.**)

Key Words: atherosclerosis ■ vulnerable plaque ■ adenovirus ■ IL-18 ■ collagen

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 IL-18. It is an IL-1 family member[3] and induces interferon (IFN)-γ, a known proatherogenic mediator,[4,5] in macrophages and in smooth muscle cells, but not in endothelial cells.[6] Binding to the IL-18 receptor results in enhanced secretion of many cytokines and proteins causally involved in atherosclerosis, among which are IL-6, IL-8, intercellular adhesion molecule-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 on stimulation with IL-1β and tumor necrosis factor-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 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 atherogenesis is well-established, its effect at later stages of plaque development and 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. IL-18 serum levels have been found to correlate to cardiovascular morbidity and mortality in patients with coronary heart disease.[10–13] In addition, Mallat et al reported elevated IL-18 mRNA levels in unstable human plaques from carotid endarterectomy.[14]

Plaque rupture is the predominant cause of acute ischemic events. The fibrous cap maintains the structural integrity of the atheromatous lesion. An imbalance in synthesis and degradation of extracellular matrix leads to thinning of the cap and renders the plaque prone to rupture. It is conceivable that IL-18 promotes this through induction of IFN-γ, apoptosis, or protease activity. Various MMPs are overexpressed in vulnerable plaques and induced by pro-inflammatory mediators.[15–17] In addition, inflammatory processes can reduce the number of intimal cells or their ability for collagen synthesis,

Original received March 29, 2004; final version accepted September 8, 2004.

From the Division of Biopharmaceutics (R.d.N., J.H.v.d.T., C.J.N.V., J.K., Th.J.C.v.B., E.A.L.B.) and the Department of Cardiology (R.d.N., J.W.J., E.E.v.d.W.), Leiden University Medical Center, Leiden, the Netherlands.

Correspondence to R. de Nooijer, LACDR, Division of Biopharmaceutics, Gorlaeus Laboratory, PO Box 9502, 2300 RA Leiden, the Netherlands. E-mail r.de.nooijer@chem.leidenuniv.nl

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000147126.99529.0a**

thereby compromising matrix production and threatening plaque stability.

Because little is known about the effects of IL-18 on the composition and stability of advanced plaques, we aimed to assess the effect of systemic IL-18 overexpression on the morphology and stability of preexisting 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 carotid artery.[18] 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 to 12 weeks of age, were obtained from our own breeding stock (Gorlaeus Laboratories, Leiden, the Netherlands). Mice were placed on a Western-type diet containing 0.25% cholesterol (Special Diets Services). 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

Atherosclerotic lesions were induced by perivascular collar placement on the common carotid artery as previously described.[18] Five weeks after surgery, animals were injected intravenously with 200 μL of an adenovirus suspension (5.0×10^9 pfu/mL) carrying a murine IL-18 (Ad.IL-18) or an empty transgene (Ad.Empty) under control of a cytomegalovirus promoter.[19] Two weeks later, lesions from both carotids were analyzed histologically (n=16 to 17) with regard to plaque morphology and composition or by real-time polymerase chain reaction (n=14).

Weekly blood samples were taken to monitor plasma cholesterol levels using enzymatic procedures (Roche Diagnostics). IL-18 levels were determined by enzyme-linked immunosorbent assay (OptEIA Set Mouse IL-18; BD Biosciences, San Diego, Calif) 1 week after injection of the adenoviral vector according to the manufacturer's instructions.

Tissue Harvesting and Preparation for Histological Analysis

One day before euthanization, phenylephrine (8 μg/kg intravenous; Sigma Diagnostics, St. Louis, Mo) was administered to all animals to asses the effect on plaque integrity via hemodynamic challenge. Cryosections prepared from carotids were stained with hematoxylin and eosin. Collagen was visualized using picro Sirius Red staining. Corresponding sections were stained immunohistochemically with antibodies directed against mouse metallophilic macrophages (clone MOMA2; Sigma) and α-SM-actin (clone 1A4; Sigma). To assess intimal cell death, sections were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using protocols provided by the manufacturer (In Situ Cell Death Detection Kit, Roche).

The site of maximal plaque size was selected for morphometry. In addition, cap and core areas were measured, as well as mean cap thickness. Stage of lesion progression was assessed using classification criteria defined by Virmani et al.[20] Of 6 categories (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 2 classes are considered stable, whereas lesions in classes 3 to 6 are perceived as plaques with characteristics of vulnerability. Thin cap atheroma are defined as plaques with fibrous caps $\leq$3 cell layers of thickness. Macrophage-positive, VSMC-positive, and collagen-positive areas were determined by computer-assisted color-gated measurement, and were related to intimal surface area. TUNEL positive cells were related to the total number of intimal cells.

Tissue Harvesting and Preparation for Expression Analysis

For expression analysis, freshly isolated plaques were pooled in 3 groups per treatment group for RNA isolation using the TriZol method (Invitrogen). Purified RNA was DNase treated and reverse-transcribed (RevertAid M-MulV Reverse Transcriptase) according to protocols provided by the manufacturer. Quantitative gene expression analysis was performed using SYBR Green technology. Primers were designed for murine α-SM-actin, Matrix Gla Protein, desmin, MMP-3, MMP-9, and MMP-13, TIMP-1, heat shock protein 47 (hsp47), and procollagen type I α_2, and validated for identical efficiencies (for sequences, please see http://atvb.ahajournals.org). Target gene mRNA levels were expressed relative to the housekeeping gene 36b4.

Detection of Protease Activity by Gelatin and Collagen Zymography

Murine hepatoma cells (mhAT3F2)[21] were plated 24 hours before infection and Ad.IL-18 or Ad.Empty was added to the cells at 500 multiplicity of infection (MOI) in 500 μL of fresh media and left for 16 hours. Cells were washed and incubated in fresh media for 24 hours before collection of conditioned media. Samples were centrifuged, added to murine VSMCs (isolated from C57Bl/6 murine aortas)[22] or RAW 264.7 cells and left for 24 hours.

To investigate proteolytic activity after incubation with conditioned media, samples were subjected to gelatin and collagen zymography. Briefly, equal sample volumes were loaded onto a polyacrylamide gel containing 0.1% sodium dodecyl sulfate and 1.5 mg/mL gelatin or 1 mg/mL collagen. After electrophoresis, gels were incubated in 2.5% Triton-X-100 for 30 minutes, in developing buffer for 16 hours, and subsequently stained with Coomassie Brilliant Blue. After destaining, bands of lysis representing protease activity were visualized as stainless spots against a blue background (for details, please see http://atvb.ahajournals.org).

Statistics

Plaque size was analyzed using the Mann–Whitney *U* test. Other morphometric parameters, plaque composition, as well as TUNEL staining and differences in change in threshold of cycle number (ΔCt) were compared using the 2-tailed Student *t* test. Correlations were determined with Spearman rank correlation test. Differences in the occurrence of adverse events and in classification were analyzed with the Yates corrected 2-sided Fisher exact test.

Results

Throughout the experiments, the mice remained in good health and adenoviral gene transfer was tolerated very well. Neither body weight nor cholesterol levels were affected by the procedures. IL-18 plasma levels were measured 1 week after infection. Intravenous administration of 1×10^9 pfu of Ad.mIL-18 led to 9-fold increase of circulating IL-18, which was raised from 150±200 pg/mL to 1350±650 pg/mL (P=0.0001).

IL-18 Overexpression Does not Affect Size of Advanced Plaques

Because the aim of this study was to assess the effect of IL-18 on preexisting plaques, it was important to induce lesions at a predefined place in a time-controlled fashion before adenoviral gene modulation. This way, neither plaque size nor location could be held accountable for changes in composi-

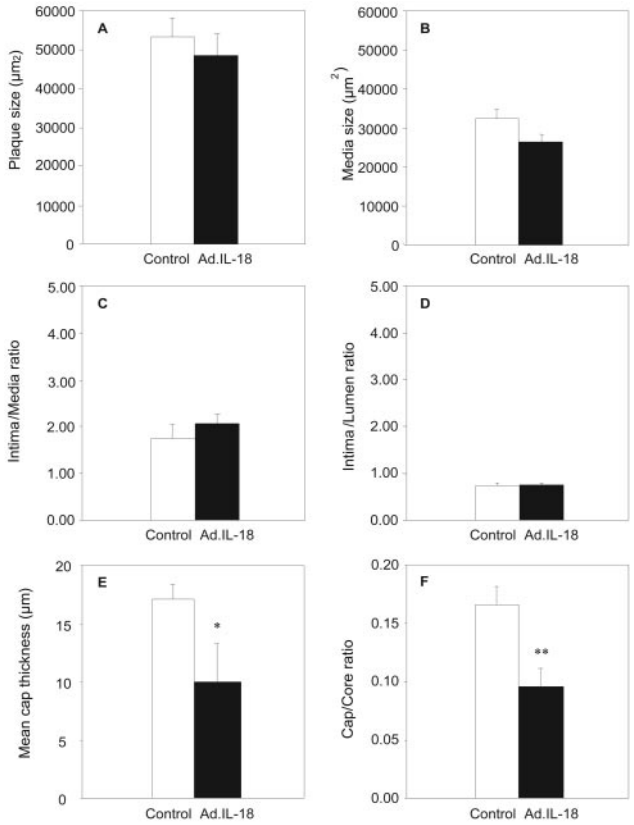

Figure 1. Baseline characteristics of plaques were comparable between groups. Two weeks after transduction, Ad.Empty-treated and Ad.IL-18–treated mice did not show any differences in morphometric parameters such as plaque size (A), media size (B), intima/media ratio (C), and intima/lumen ratio (D). Mean cap thickness differed significantly between groups (E), as did the cap/core ratio (F). *P=0.026, **P=0.002. Values are mean±SEM.

tion or stability. For this reason, we applied the collar model for rapid atherogenesis, in which placement of a perivascular collar on the common carotid artery induces plaques within 4 to 6 weeks. Five weeks after surgery, the animals were injected intravenously with the adenoviral vectors, and 2 weeks later the carotids were harvested for further analysis.

No difference in plaque size was detected between groups (Ad.IL-18: 49 000±5000 μm^2 versus Ad.Empty: 53 000±5000 μm^2), and we did not find any difference in intima/lumen ratios (Figure 1A and 1D). Media size and intima/media ratios did not differ between groups (Figure 1B and 1C), suggesting that IL-18 did not affect outward remodeling.

Systemic IL-18 Overexpression Leads 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 applied the classification as described by Virmani.[20] Fibrous lesions and atheromatous plaques, class 1 to 2, were perceived as stable. Plaques showing thin cap morphology or adverse events, like intraplaque hemorrhage or intramural thrombosis (classes 3 to 6), were considered unstable. In the Ad.IL-18–treated

Distribution of Collar-Induced Lesions Showing Stable or Vulnerable Plaque Morphology

	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

IL-18–treated mice showed higher prevalence of unstable plaques (62% vs. 24% in controls, P=0.037).

group, 62% of the lesions displayed features of vulnerable plaque morphology as compared with only 24% in the controls (P=0.037) (Table). Adverse events (class 5), in this case intralesional bleeding (an established sign of plaque vulnerability), were observed in 19% of IL-18 overexpressing plaques but not in control plaques (Figure 2). Plaque rupture or erosion could not be detected. To confirm the higher incidence of thin cap atheroma, fibrous cap thickness was measured at 12 evenly spaced sites of the cap. Mean cap thickness decreased 41% after IL-18 exposure (Ad.Empty: 17.0±1.5 μm versus Ad.IL-18: 9.9±3.0 μm; P=0.026) (Figure 1E). Likewise, cap area (Ad.Empty: 7720±480 μm^2 versus Ad.IL-18: 4190±750 μm^2; P=0.0002) and cap/core ratio (Figure 1F) (Ad.Empty: 0.17±0.01 versus Ad.IL-18: 0.10±0.02; P=0.002) decreased significantly.

Effects on Plaque Composition

The IL-18 group showed a 44% decrease in intimal collagen content (collagen/intima ratio, 0.22 versus 0.39; P=0.003) (Figure 3 A to C). Because lesions had already progressed to an advanced stage in both groups, it is unlikely that the

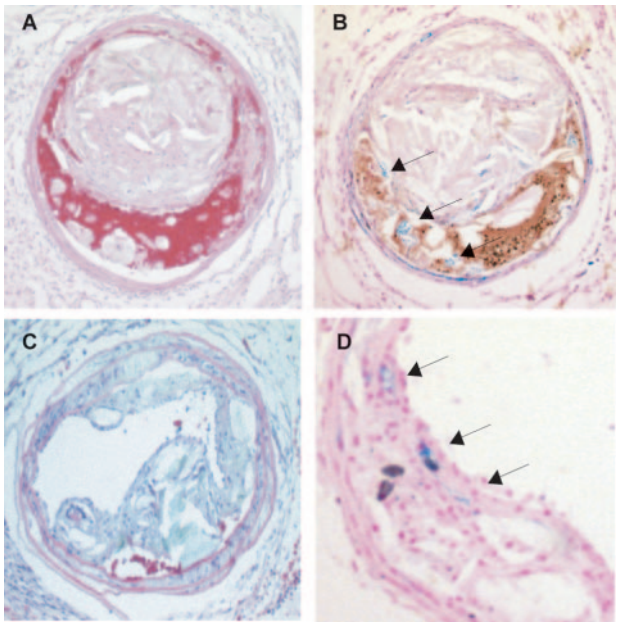

Figure 2. Massive intraplaque hemorrhage on hematoxylin and eosin staining (A, C) and Perl staining for iron (B, D). Arrows show deposition of iron, the product of degraded hemoglobin, suggestive for the presence of intramural thrombi.

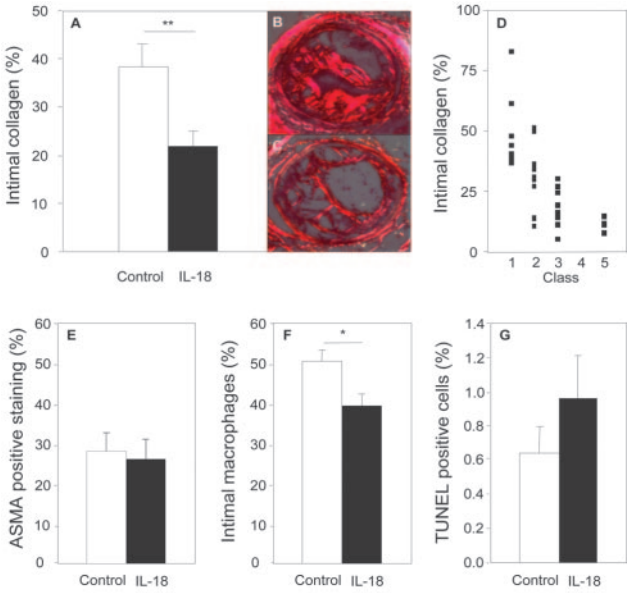

Figure 3. Plaque composition. A, Intimal collagen (%). 40% decrease in IL-18 treatment group. Picro Sirius red-stained sections from controls (B) and Ad.IL-18–treated mice (C). D, Correlation between intimal collagen and morphological class ($R=-0.79$, $P<0.0001$). E, Intimal smooth muscle cells. Percentage of alpha smooth muscle actin (ASMA) positive staining. F, Percentage of intimal macrophages. G, Percentage of TUNEL-positive cells. Values are mean±SEM. *$P=0.009$, **$P=0.003$.

observed depletion of collagen already existed at the time of IL-18 introduction. Low collagen content was mainly observed in unstable plaques and thus largely class-dependent ($R=-0.79$, $P<0.0001$) (Figure 3D). Still, although not reaching 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.

Cell density may reflect the intimal capacity to produce matrix components. For this reason, we quantified the number of cells relative to intimal area, showing that IL-18 treatment moderately decreased intimal cell density (0.0044 ± 0.0013 versus 0.0057 ± 0.0014 nuclei/μm^2 in controls; $P=0.044$). However, VSMC content as determined with α-actin staining did not differ between groups (Ad.IL-18–treated: 26% versus Ad.Empty–treated: 28%; $P=0.76$) (Figure 3E). Interestingly, a decrease in the relative macrophage content of the lesion was observed in Ad.IL-18–treated mice (39% versus 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 3F). The latter also might explain the reduction in cell density. Finally, the rate of apoptosis was assessed with 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 3G).

Effects on Gene Expression Within the Lesion

To elucidate the mechanism of the observed collagen reduction, we performed real-time polymerase chain reaction analysis on lesional mRNA. This did not reveal an altered gene expression of markers for synthetic VSMCs phenotype, like Matrix Gla Protein, or of those for the contractile phenotype (eg, desmin). Also, a difference in expression of the $\alpha 2$ chain of procollagen type I, the major collagen constituent of the plaque, and the chaperone hsp47, involved in collagen processing,[23,24] could not be detected (Figure 4). These data suggest that the rate of collagen synthesis remained unaltered after IL-18 exposure and that processes other than phenotypic modulation or collagen production might be underlying the observed decrease in collagen content.

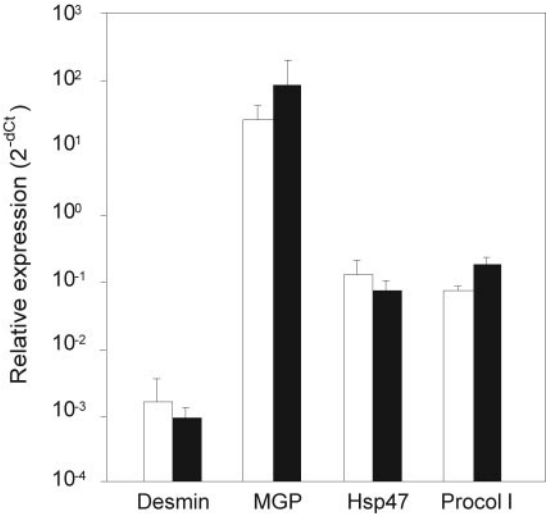

Figure 4. Intralesional gene expression levels for VSMC phenotype and collagen synthesis. None of these changed significantly after IL-18 introduction. Values are mean±SD.

IL-18 Causes Increased Proteolytic Activity of VSMCs In Vitro

Because procollagen type I expression and VSMC number did not decrease, and because phenotype did not seem to change on 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 24 hours after removal of the virus, and murine VSMCs and RAW 264.7 cells were incubated with this media for 24 hours. RAW 264.7 cells were used to exclude paracrine effects that may result from lymphoid contamination complicating isolation of primary macrophages.[25] Zymographic analysis of the 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 controls versus 108.0 ± 9.1 INT×mm^2 in Ad.IL-18–treated group; $P=0.0003$) (Figure 5A). No effect could be seen on the activity of the gelatinases MMP-2 (Figure 5B) and MMP-9 (not shown).

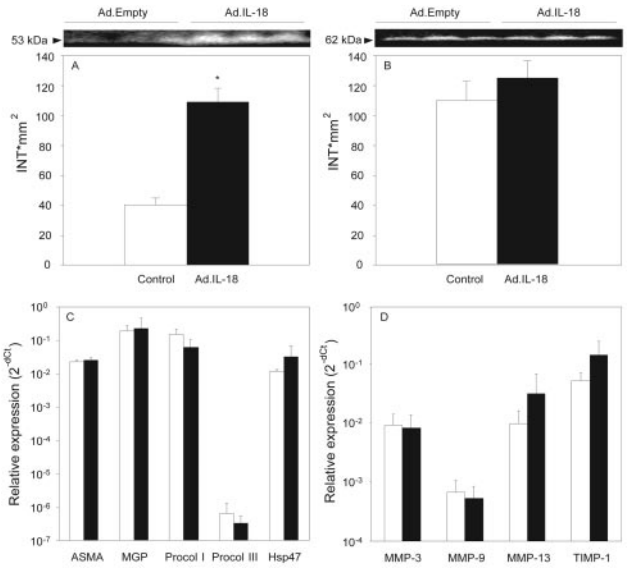

Figure 5. A, Collagen zymography. Conditioned media from Ad.IL-18–transduced hepatoma cells caused an increase in collagenolytic activity in VSMCs at 53 kDa. *P=0.0003. B, Gelatin zymography. No change could be detected in MMP-2 (62kDa) and MMP-9 activity. Values are mean±SEM. C and D, Gene expression in VSMCs after incubation with the conditioned media samples. Collagen synthesis (C) and MMP expression (D) are unchanged after IL-18 treatment. Values are mean±SD.

In line with the in vivo observations, expression levels of genes indicating VSMC phenotype and genes involved in collagen synthesis were unaffected (Figure 5C). Also, MMP and tissue inhibitor of metalloproteinases (TIMP)-1 mRNA levels remained unchanged on IL-18 treatment (Figure 5D), suggesting that IL-18 did not alter the expression or inhibition of MMP-13, but rather its activation.

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.[7] In our study, we used female mice. Intriguingly, IFN-γ deficiency did not attenuate atherogenesis in females[26] to a similar extent as it did in males; therefore, it seems less likely that the observed destabilizing effect of IL-18 is IFN-γ–dependent. Although several epidemiological reports suggest an involvement of IL-18 in plaque rupture and acute coronary events,[9–11,14,27] studies addressing its role in plaque stability are surprisingly lacking and no evidence of causality has been shown to this date. Our observations, for the first time to our knowledge, 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 an IL-18 transgene caused an elevation of circulating IL-18. Because the size of the preexisting 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 progression. Overexpression of IL-18 caused a marked decrease in intimal collagen and led to a plaque phenotype with clear characteristics of vulnerability. In 3 IL-18–treated vessels, intraplaque hemorrhage was observed, accompanied with iron deposits, reflective of intramural thrombi. Collagen type I expression, the amount of VSMCs, and the incidence of apoptosis had not 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 III,[28] the homeostasis of which is maintained at various levels. Collagen synthesis within the plaque is mainly attributable to VSMCs[29] and modulated by various growth factors and cytokines, either directly by influencing procollagen expression or indirectly by shifting 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 collagen synthesis.[30]

In our study, procollagen type I mRNA levels did not decrease at the site of the lesion and the expression of desmin and Matrix Gla Protein remained constant. Therefore, it is unlikely that collagen synthesis was diminished, either by transcriptional downregulation or by phenotypic modulation. Also, the expression of hsp47, an important chaperone in intracellular 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.[31,32] Earlier observations in our laboratory indicate that induction of VSMC apoptosis in the fibrous cap will decrease cell density, resulting in cap thinning and plaque destabilization.[33] Recently, IL-18 has been shown to promote apoptosis by stimulating the secretion of FasL and the expression of tumor necrosis factor receptor I (TNFR-I).[34,35] Although in this study a slight increase in apoptosis could be noticed in the IL-18 treatment group, it did not reach statistical significance. Furthermore, α-SM-actin staining showed a very moderate, nonsignificant 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. The VSMCs, as well as macrophages, secrete a wide array of proteases and protease inhibitors. In particular, MMPs have been associated with both atherogenesis and plaque destabilization.[15,16,36–38] Gerdes et al reported a stimulatory effect of IL-18 on MMP-1, MMP-9, and MMP-13 protein expression in human macrophages,[6] whereas targeted deletion of caspase-1 not only decreased IL-18 production but also decreased MMP-3.[39] Each of these proteases was shown to be highly expressed in unstable plaques.[16,17]

In our study, we tested the effect of IL-18 exposure on VSMCs and macrophages. Because even a minor contamination with lymphoid cells during primary monocyte/mac-

rophage isolation can result in a paracrine activation of these macrophages,[25] 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 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,[40] this study showed that VSMCs can also play a part in cap thinning and plaque destabilization. Because MMP-13 and TIMP-1 expression were barely affected, it seems that IL-18 enhanced the activation of MMP-13 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. Further investigations concerning IL-18–related effects on MMP-13 regulation, however, are mandatory for a better understanding of its matrix modulating properties.

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 toward vulnerable plaque morphology. This proteolytic activity may be executed by VSMCs, which might attenuate the importance of macrophage infiltration as a condition sine qua non for thinning of the fibrous cap. In conclusion, these data underline the importance of IL-18 in extracellular matrix integrity and in plaque stability, making it an attractive target for therapeutic intervention.

Acknowledgments

This work was supported by the Netherlands Heart Foundation (grant no. M93.001 [to R.N., J.T.], 2000T40 [to J.K.], 2001D032 [to J.W.J.], and 2004T201 [to E.B.]) and the Netherlands Organization for Scientific Research (NWO; VENI award [to J.T.] and ZonMw 016026019 [to E.B.]). We thank Dr Shoshana Levy from Stanford University for supplying us with the adenoviral vectors.

References

1. Ross R. Atherosclerosis–an inflammatory disease. *N Engl J Med*. 1999; 340:115–126.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105:1135–1143.
3. Dinarello CA. Interleukin-18, a proinflammatory cytokine. *Eur Cytokine Netw*. 2000;11:483–486.
4. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-γ potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest*. 1997; 99:2752–2761.
5. Whitman SC, Ravisankar P, Elam H, Daugherty A. Exogenous interferon-γ enhances atherosclerosis in apolipoprotein E−/− mice. *Am J Pathol*. 2000;157:1819–1824.
6. Gerdes N, Sukhova GK, Libby P, Reynolds RS, Young JL, Schonbeck U. Expression of interleukin (IL)-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for atherogenesis. *J Exp Med*. 2002;195:245–257.
7. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein E(−/−) mice through release of interferon-γ. *Circ Res*. 2002;90:E34–E38.
8. Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res*. 2003;59: 234–240.
9. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. *Circ Res*. 2001;89:E41–E45.
10. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, Rupprecht HJ. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina. *Circulation*. 2002;106:24–30.
11. Mallat Z, Henry P, Fressonnet R, Alouani S, Scoazec A, Beaufils P, Chvatchko Y, Tedgui A. Increased plasma concentrations of interleukin-18 in acute coronary syndromes. *Heart*. 2002;88:467–469.
12. SoRelle R. Interleukin-18 predicts coronary events. *Circulation*. 2003; 108:e9051–e9065.
13. Blankenberg S, Luc G, Ducimetiere P, Arveiler D, Ferrieres J, Amouyel P, Evans A, Cambien F, Tiret L. Interleukin-18 and the risk of coronary heart disease in European men: the Prospective Epidemiological Study of Myocardial Infarction (PRIME). *Circulation*. 2003;108:2453–2459.
14. Mallat Z, Corbaz A, Scoazec A, Besnard S, Leseche G, Chvatchko Y, Tedgui A. Expression of interleukin-18 in human atherosclerotic plaques and relation to plaque instability. *Circulation*. 2001;104:1598–1603.
15. 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.
16. Sukhova GK, Schonbeck U, Rabkin E, Schoen FJ, Poole AR, Billinghurst RC, Libby P. Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation*. 1999;99:2503–2509.
17. Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PR, Thompson MM. Increased matrix metalloproteinase-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. *Stroke*. 2000;31:40–47.
18. von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation*. 2001;103:1164–1170.
19. Walter DM, Wong CP, DeKruyff RH, Berry GJ, Levy S, Umetsu DT. Il-18 gene transfer by adenovirus prevents the development of and reverses established allergen-induced airway hyperreactivity. *J Immunol*. 2001;166:6392–6398.
20. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2000; 20:1262–1275.
21. Antoine B, Levrat F, Vallet V, Berbar T, Cartier N, Dubois N, Briand P, Kahn A. Gene expression in hepatocyte-like lines established by targeted carcinogenesis in transgenic mice. *Exp Cell Res*. 1992;200:175–185.
22. Michon IN, Hauer AD, von der Thusen JH, Molenaar TJ, van Berkel TJ, Biessen EA, Kuiper J. Targeting of peptides to restenotic vascular smooth muscle cells using phage display in vitro and in vivo. *Biochim Biophys Acta*. 2002;1591:87–97.
23. Rocnik E, Saward L, Pickering JG. HSP47 expression by smooth muscle cells is increased during arterial development and lesion formation and is inhibited by fibrillar collagen. *Arterioscler Thromb Vasc Biol*. 2001;21: 40–46.
24. Murakami S, Toda Y, Seki T, Munetomo E, Kondo Y, Sakurai T, Furukawa Y, Matsuyama M, Nagate T, Hosokawa N, Nagata K. Heat shock protein (HSP) 47 and collagen are upregulated during neointimal formation in the balloon-injured rat carotid artery. *Atherosclerosis*. 2001; 157:361–368.
25. Golab J, Zagozdzon, Stoklosal T, Kaminski R, Kozar K, Jakobisiak M. Direct stimulation of macrophages by IL-12 and IL-18–a bridge too far? *Immunol Lett*. 2000;72:153–157.
26. Whitman SC, Ravisankar P, Daugherty A. IFN-γ deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E−/− mice. *J Interferon Cytokine Res*. 2002;22:661–670.
27. Yamashita H, Shimada K, Seki E, Mokuno H, Daida H. Concentrations of interleukins, interferon, and C-reactive protein in stable and unstable angina pectoris. *Am J Cardiol*. 2003;91:133–136.
28. Andreeva ER, Pugach IM, Orekhov AN. Collagen-synthesizing cells in initial and advanced atherosclerotic lesions of human aorta. *Atherosclerosis*. 1997;130:133–142.
29. Pietila K, Nikkari T. Role of the arterial smooth muscle cell in the pathogenesis of atherosclerosis. *Med Biol*. 1983;61:31–44.
30. 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.
31. Geng YJ, Henderson LE, Levesque EB, Muszynski M, Libby P. Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 1997;17:2200–2208.
32. Kockx MM, Knaapen MW. The role of apoptosis in vascular disease. *J Pathol*. 2000;190:267–280.
33. von der Thusen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, Biessen EA. Induction of atherosclerotic plaque rupture in apolipoprotein E−/− mice after adenovirus-mediated transfer of p53. *Circulation*. 2002;105:2064–2070.
34. Micallef MJ, Tanimoto T, Torigoe K, Nishida Y, Kohno K, Ikegami H, Kurimoto M. Simultaneous exposure to interleukin-18 and interleukin-10 in vitro synergistically augments murine spleen natural killer cell activity. *Cancer Immunol Immunother*. 1999;48:109–117.
35. Marino E, Cardier JE. Differential effect of IL-18 on endothelial cell apoptosis mediated by TNF-α and Fas (CD95). *Cytokine*. 2003;22:142–148.
36. Rajavashisth TB, Xu XP, Jovinge S, Meisel S, Xu XO, Chai NN, Fishbein MC, Kaul S, Cercek B, Sharifi B, Shah PK. Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators. *Circulation*. 1999;99: 3103–3109.
37. Loftus IM, Naylor AR, Bell PR, Thompson MM. Matrix metalloproteinases and atherosclerotic plaque instability. *Br J Surg*. 2002;89: 680–694.
38. Shah PK, Galis ZS. Matrix metalloproteinase hypothesis of plaque rupture: players keep piling up but questions remain. *Circulation*. 2001; 104:1878–1880.
39. Frantz S, Ducharme A, Sawyer D, Rohde LE, Kobzik L, Fukazawa R, Tracey D, Allen H, Lee RT, Kelly RA. Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction. *J Mol Cell Cardiol*. 2003;35:685–694.
40. Shah PK, Falk E, Badimon JJ, Fernandez-Ortiz A, Mailhac A, Villareal-Levy G, Fallon JT, Regnstrom J, Fuster V. Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. *Circulation*. 1995;92: 1565–1569.

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.

Table I. PCR primersets.

Gene	Forward primer	Reverse primer
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
(alpha)-Actin	TCCCTGGAGAAGAGCTACGAACT	GATGCCCGCTGACTCCAT
CD68 (Macrosialin)	CCTCCACCCTCGCCTAGTC	TTGGGTATAGGATTCGGATTTGA
Desmin	GATGCAGCCACTCTAGCTCGTATT	CTCCTCTTCATGCACTTTCTTAAGG
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
Hsp47	ACAAGATGCGAGATGAGTTGTAGAGT	TAGCACCCATGTGTCTCAGGAA
IL-18	ACACAACAAGATGGAGTTTGAATCTTC	TTTCAGAATGAGTTTGAAAGCATCA
IL1Beta	TGG TGT GTG ACG TTC CCA TTA	AGG TGG AGA GCT TTC AGC TCA TAT
MGP	GCATGTGTTGCTTGCTCCTTAC	TCATTACTTTCAACCCGCAGAA
MMP-3	TTTAAAGGAAATCAGTTCTGGGCTATAC	CGTAAGTGTGGGACCCAGAC
MMP-9	CTGGCGTGTGAGTTTCCAAAAT	TGCACGGTTGAAGCAAAGAA
MMP-13	CAACCTATTCCTGGTTGCTGC	ATCAGAGCTTCAGCCTTGGC
Osteopontin	CAGGCATTCTCGGAGGAAC	GAGCTGGCCAGAATCAGTCACTTT
Procol I (Cola2)	TGTACTATGGATGCCATCAAAGTGT	CCATTGATAGTCTCTCCTAACCAGACA
Procol III (Cola2)	TGCCCAACTGCGCTTCA	CCAGCCTGACAGGTTGGAAA
TIMP-1	ACACCCCAGTCATGGAAAGC	CTTAGGCGGCCCGTGAT