Integrin-Mediated Transcriptional Activation of Inhibitor of Apoptosis Proteins Protects Smooth Muscle Cells Against Apoptosis Induced by Degraded Collagen

Karin von Wnuck Lipinski, Petra Keul, Nicola Ferri, Susann Lucke, Gerd Heusch, Jens W. Fischer, Bodo Levkau

Abstract**—**Apoptosis of smooth muscle cells (SMC) and degradation of the extracellular matrix (ECM) have both been implicated in atherosclerotic plaque rupture. We have previously reported that degraded type I collagen fragments induce a rapid but transient apoptotic burst initiated by calpains in SMC. The aim of the current study was to identify the pathway responsible for consecutive SMC survival. We show that exposure of SMC to collagen fragments resulted in a sustained activation of nuclear factor (NF)-κB via phosphorylation and degradation of IκBα. Its prevention through retroviral expression of superrepressor IκBα or proteasome inhibition potently induced apoptosis. In the presence of blocking antibodies to $\alpha_v\beta_3$ integrin and RGD peptides, collagen fragments no longer activated NF-κB and apoptosis was enhanced. The mechanism by which NF-κB was protecting SMC against collagen fragment-induced apoptosis was a transcriptional activation of several endogenous caspase inhibitors of the inhibitor of apoptosis protein (IAP) family as: (1) the expression of xIAP, c-IAP2, and survivin was potently induced by collagen fragments; (2) IAP expression was abrogated by superrepressor IκBα; and (3) knockdown of each of the 3 IAPs by small interfering RNA (siRNA) resulted in enhanced apoptosis after collagen fragment treatment. Our data suggest that SMC exposed to degraded collagen are protected against apoptosis by a mechanism involving $\alpha_v\beta_3$-dependent NF-κB activation with consequent activation of IAPs. This may constitute a novel antiapoptotic pathway ensuring SMC survival in settings of enhanced ECM degradation such as cell migration, vascular remodeling, and atherosclerotic plaque rupture. **(*Circ Res*. 2006;98:1490-1497.)**

Key Words: apoptosis ■ caspase activation ■ NF-κB ■ vascular smooth muscle cells ■ collagen ■ extracellular matrix ■ atherosclerosis

Downloaded from http://ahajournals.org by on September 27, 2019

Extracellular matrix (ECM) remodeling plays a major role in the pathogenesis of atherosclerosis and restenosis.[1] Smooth muscle cells (SMC) both synthesize and degrade the surrounding ECM,[2] the composition and integrity of which is viewed as a crucial determinant of plaque stability. The major ECM components synthesized and deposited by SMC in the plaque are type I and III collagens.[3] These collagens are proteolytic substrates for several metalloproteinases (MMPs) such as MMP-1, -2, -8, -9, and -13 and MT1-MMP that are produced and secreted by plaque SMC and macrophages. Although the degradation of fibrous cap collagens has been widely implied in plaque rupture through its mere mechanical destabilization,[4] little is known about the biological role of the resulting collagen degradation products. As collagen degradation and SMC apoptosis coincide at sites of plaque rupture and have both been implied in its pathogenesis,[5,6] we hypothesized that there may be a link between the 2 processes. Collagen degradation products are by no means inert in respect to apoptosis: in endothelial cells, proteolytic fragments of type IV collagen (endostatin) and XVIII collagen (tumstatin) potently induce apoptosis,[7,8] whereas in rat vascular SMC, type I collagen fragments inhibit apoptosis through the production of the survival factor tenascin-C.[9]

Our previous studies have shown that extended culture of human SMC on polymerized type I collagen profoundly altered their focal contacts through calpain-mediated proteolysis of focal adhesion proteins such as FAK, talin, and paxillin and that this process was mediated by MMP-generated collagen fragments.[10] Recently, we demonstrated that the identical collagen fragments lead to a rapid activation of caspases and apoptosis that was dependent on calpains.[11] This coincided with calpain-dependent proteolysis of X chromosome–linked inhibitor of apoptosis protein (xIAP) resulting in loss of its caspase-inhibitory potential.[11] How-

Original received December 5, 2005; resubmission received March 29, 2006; revised resubmission received May 1, 2006; accepted May 17, 2006.

From the Institute of Pathophysiology (K.v.W.L., P.K., S.L., G.H., B.L.), Center of Internal Medicine, University Hospital, Essen, Germany; Department of Pharmacological Sciences (N.F.), University of Milan, Italy; and Molecular Pharmacology (J.W.F.), Institute of Pharmacology and Clinical Pharmacology, Heinrich-Heine University Düsseldorf, Germany.

Correspondence to Bodo Levkau, Institute of Pathophysiology, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany. E-mail levkau@uni-essen.de

© 2006 American Heart Association, Inc.

Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000229267.77982.0d**

ever, caspase activation was moderate, xIAP destruction transient and most of the SMC survived. This led us to postulate the existence of an antiapoptotic pathway activated in parallel by type I collagen fragments and dominant over the apoptotic. In the present study, we have identified this survival pathway as the activation of the transcription factor nuclear factor (NF)-κB by type I collagen fragments with consecutive activation of several members of the inhibitor of apoptosis (IAP) family. We have characterized the necessity and sufficiency of the IAPs for SMC survival in a proteolyzed collagen environment and have identified the responsible integrins.

Materials and Methods

Reagents and Antibodies

The following antibodies were used for immunoblotting: xIAP, poly(ADP-ribosyl) polymerase (PARP) (BD Biosciences), c-IAP2, IκBα (Santa Cruz Biotechnology); p-IκBα, cleaved caspase-3, cleaved lamin A (New England Biolabs); survivin (Novus Biologicals); caspase-3 (R&D Systems); α- and β-actin (Sigma-Aldrich); and GAPDH (HyTest Ltd). Other reagents used were: secondary antibodies (Vector Laboratories); blocking antibody against $\alpha_v b_3$ (LM609; Chemicon); mouse IgG_1 (BD Biosciences); RGD peptides (Invitrogen); *N*-acetyl-leucinyl-leucinyl-norleucinal (LLnL) and Z-Leu-Leu-Leu-aldehyde (MG-132; Alexis); and cycloheximide (Sigma-Aldrich).

SMC Culture and Preparation of Collagen Fragments

Human newborn arterial SMC from the thoracic aorta were a kind gift of Elaine W. Raines, University of Washington, Seattle, and were used in all our previous studies on collagen fragments.[10,11] Type I collagen gels (1.0 mg/mL final collagen concentration) were prepared by neutralizing the bovine skin collagen solution (Vitrogen 100, Cohesion) with one-sixth volume of a 7-fold DMEM and polymerization at 37°C for 12 hours. Collagen-coated dishes were prepared by incubating 0.1 mg/mL collagen solution in 0.1 mol/L acetic acid at 37°C for 12 hours. Degraded type I collagen was prepared as described[10,11] by incubating polymerized collagen gels with 2 mg/mL collagenase type 3 (Worthington Biochemical Corp) at 37°C for 30 minutes and addition of an equal volume of 10% FCS/DMEM.

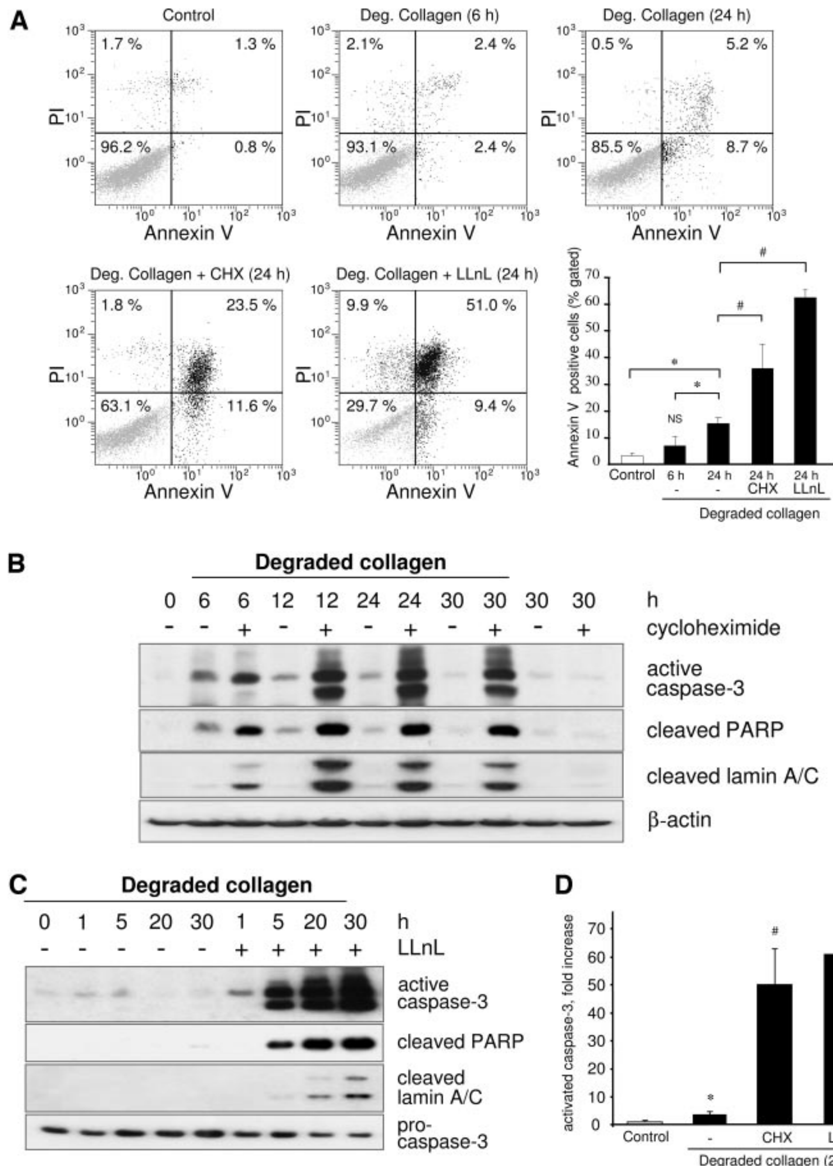

Figure 1. Degraded collagen fragments induce apoptosis in human SMC in the presence of cycloheximide and proteasome inhibition, respectively. A, SMC were exposed to degraded type I collagen fragments (250 μg/mL) in the presence and absence of 10 μg/mL cycloheximide or 50 μmol/L LLnL for the indicated times. Apoptosis was assessed by flow cytometric analysis after double staining with FITC-labeled annexin V and PI. One representative experiment of 3 is shown. Bar graphs represent mean values±SD from all experiments. ANOVA was used to compare 24 hours of collagen fragment treatment with 6 hours and control.*$P<0.05$ vs untreated controls. In a separate ANOVA, 24 hours of collagen fragment treatment with and without inhibitors was compared. #$P<0.05$ vs SMC incubated with collagen fragments without inhibitors for 24 hours. NS indicates not significantly different from control. B and C, Apoptosis was evaluated by Western blot analysis using antibodies against active caspase-3, PARP, and cleaved lamin A/C. Expression of β-actin or pro–caspase-3 were used as loading controls. Shown are representative experiments of 4. D, Quantification of caspase-3 activation is represented as fold increase after densitometric analysis by NIH image. Results are the mean±SD of 4 independent experiments. Statistical analysis was performed as in Figure 1A. *$P<0.05$ vs untreated controls. #$P<0.05$ vs SMC incubated with collagen fragments without inhibitors for 24 hours.

Downloaded from http://ahajournals.org by on September 27, 2019

Protein Analysis

Cells were lysed in 50 mmol/L Tris-HCl, pH 8.0, 250 mmol/L NaCl, 5 mmol/L EDTA, pH 8.0, 50 mmol/L NaF, 0.5% Nonidet P-40 [NP-40], 0.5 mmol/L phenyl-methylsulfonyl fluoride, 0.5 mmol/L Na_3VO_4, and 5 μg/mL aprotinin, leupeptin, and pepstatin for 15 minutes on ice. Cell lysates were cleared by centrifugation at 15 000*g* for 5 minutes and protein concentrations determined using the BCA protein assay (Pierce). Cell proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), immunoblotted and visualized by ECL (Amersham Biosciences).

Fluorescence-Activated Cell Sorting Analysis

SMC were trypsinized, pooled with all detached cells from their respective supernatants, and washed twice with ice-cold PBS. Double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) was performed according to the protocol of the manufacturer (Beckman Coulter). Samples were analyzed in a flow cytometer (Epics XL; Beckman Coulter) using the software Expo32 ADC Analysis. For each determination, a minimum of 10 000 events per sample was acquired.

Electrophoretic Mobility Shift Assay

SMC were plated on collagen 24 hours before treatment, and electrophoretic mobility shift assays (EMSAs) were performed as previously described.[12] A double-stranded oligonucleotide containing the DNA-binding site for NF-κB (5′AGTTGAGGGGACTTTC-CCAGGC3′; Santa Cruz Biotechnology) was end-labeled using [γ^{32}P]ATP (ICN) and T4-PNK (MBI Fermentas). The lysed nuclei were centrifuged for 5 minutes at 4°C 12 000*g* and diluted with 30 μL of Buffer D (20 mmol/L Hepes, pH 8.0, 50 mmol/L KCl, 20% glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride [PMSF], and 0.5 mmol/L dithiothreitol [DTT]). Ten micrograms of nuclear extracts were mixed with 60 000 to 80 000 cpm of labeled probe in binding buffer (10 mmol/L Tris HCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.05% NP-40, pH 7.5) and 50 μg/mL poly dIdC and incubated at room temperature for 20 minutes. For competition and supershift studies, unlabeled NF-κB oligonucleotides or unspecific $p53^{mut}$ oligonucleotides and antibodies against the p50 and p65 subunits of NF-κB (Santa Cruz Biotechnology), respectively, were used.

Retroviral Infection

Human IκB$\alpha^{Ser32,36/Ala}$ was generated as previously described.[13] Retroviral expression plasmids were constructed using the internal ribosomal entry site (IRES) vector pBMN-IRES-PURO expressing the puromycin resistance gene PAC as a selectable second cistron gene.[14] Amphotrophic retrovirus was prepared using Phoenix-A packaging cells and transfections performed as described previously.[14] For selection, the retroviral supernatant was replaced with growth medium containing puromycin (5 to 10 μg/mL) 12 hours after transfection. Cells were cultured for additional 48 hours before use.

Transfection of Small Interfering RNA

The small interfering RNAs (siRNAs) with the following sense and antisense sequences were (1) for xIAP: sense, 5′-GUGGUAGUCC-UGUUUCAGCdTdT-3′; and antisense, 5′-GCUGAAACAGGACU-ACCACdT dT-3′; (2) for c-IAP2: sense, 5′-GUUCAAGCCAGUU-ACCCUCdTdT-3′; and antisense 5′-GAGGGUAACUGGCUUGA-ACdTdT-3′; (3) for survivin: sense, 5′-GAGCCAAGAACAAAA-UUGCdTdT-3′; and antisense, 5′-GCAAUUUUGUUCUUGGCU-CdTdT-3′; (4) for control (nonsilencing): sense, 5′-UUCUCCGAA-CGUGUCACGUdTdT-3′; and antisense, 5′-ACGUGACACGU-UCGGAGAAdTdT-3′. All siRNAs were purchased from Qiagen, and transfections were performed using a transfection system (Bio-Rad Laboratories) according to the protocol of the manufacturer.

Statistics

Results are given as means±SD. Comparisons between multiple groups were performed using 1-way ANOVA followed by a Fisher's least-significant difference (LSD) post hoc test. Single comparisons were made using Student *t* test. A probability value of $P<0.05$ was considered statistically significant.

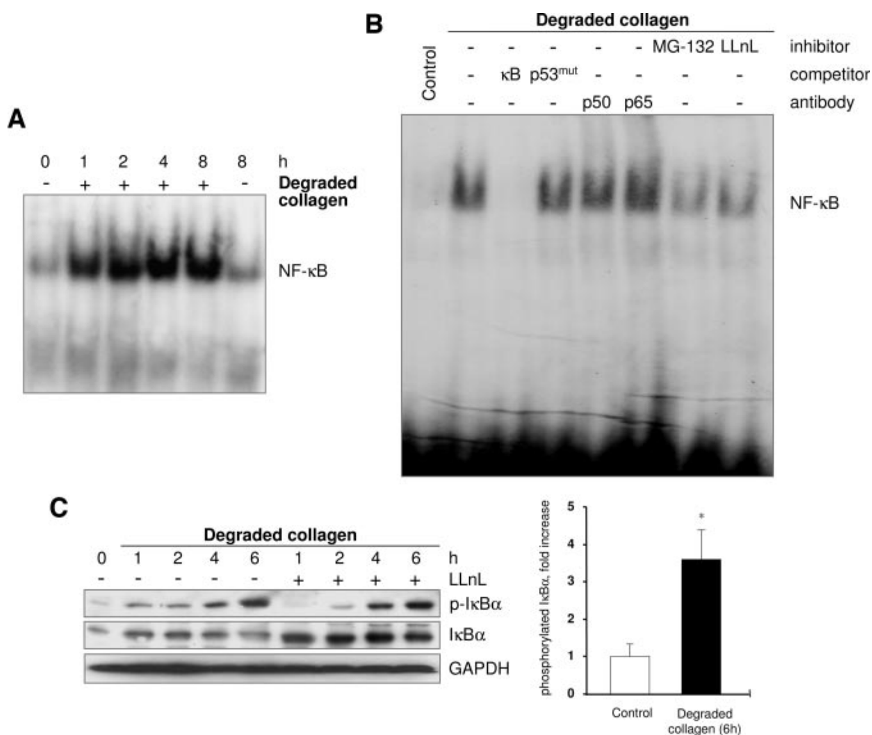

Figure 2. Degraded collagen fragments activate NF-κB in human SMC. A, Cells were incubated with or without 250 μg/mL degraded type I collagen for the indicated times, and NF-κB activity was determined by EMSA. B, To confirm specificity of NF-κB DNA binding, excess unlabeled specific κB oligonucleotide or unspecific ($p53^{mut}$) oligonucleotide were added to nuclear extracts of SMC treated with degraded collagen for 2 hours. Supershift analysis was performed using antibodies against the p50 and p65 subunits of NF-κB. The proteasomal inhibitors MG-132 (10 μmol/L) and LLnL (50 μmol/L) were added 30 minutes before the collagen fragments. C, Left, SMC were treated with or without LLnL (50 μmol/L) in the presence of collagen fragments for the indicated times and immunoblotted for phospho-IκBα(Ser32,36) and total IκBα. GAPDH was used as a loading control. Right, Quantification of phosphorylated IκBα as fold increase after treatment with collagen fragments for 6 hours (n=3 experiments). **P*<0.05 vs control.

Downloaded from http://ahajournals.org by on September 27, 2019

Results

Degraded Collagen Fragments Induce Apoptosis in SMC in the Presence of Cycloheximide or Proteasome Inhibition

We have recently shown that proteolytic fragments derived from polymerized type I collagen gels rapidly activate caspases and induce apoptosis in SMC and that this process is dependent on calpains.[11] Nevertheless, the majority of SMC recovered and survived, with only 15.4±1.9% of the cells having died after 24 hours of incubation with 250 μg/mL type I collagen fragments (Figure 1A). If, however, cycloheximide or the proteasome inhibitor LLnL were added, apoptosis was dramatically enhanced to 36.0±9.2% and 62.5±2.9%, respectively (Figure 1A), with potent activation of caspase-3 and distinct cleavage of the caspase substrates PARP and lamin A/C (Figure 1B and 1C). Thus we hypothesized that collagen fragments activate simultaneously a death and a survival pathway and tested whether the transcription factor NF-κB, known to promote survival in many experimental systems, was involved.

Degraded Collagen Fragments Induce NF-κB Activation in SMC

EMSAs for NF-κB showed a marked activation 1 hour after exposure to 250 μg/mL degraded collagen fragments that remained constant up to 8 hours (Figure 2A) and declined after 16 hours (data not shown). The NF-κB DNA binding activity was specific as it became undetectable in the presence of excess unlabeled specific oligonucleotide and was unaltered by excess unspecific oligonucleotide (Figure 2B). Preincubation of SMC with 2 different proteasome inhibitors (LLnL and MG-132) abolished NF-κB activation by degraded collagen fragments (Figure 2B). To test whether NF-κB was activated via phosphorylation and proteasomal degradation of its inhibitor IκBα[15] in our system, SMC were exposed to degraded collagen in the presence or absence of LLnL, and the levels of total and phosphorylated (Ser32,36) IκBα were examined by Western blotting. Incubation with degraded collagen fragments alone led to a rapid IκBα phosphorylation and degradation (Figure 2C). In the presence of LLnL, IκBα was also phosphorylated but accumulated over time, suggesting that NF-κB activation is mediated by proteasomal IκBα degradation (Figure 2C).

NF-κB Inhibition by Proteasomal Inhibitors and a Superrepressor IκBα$^{Ser32,36/Ala}$ Induces Apoptosis in SMC Exposed to Degraded Collagen

Because activation of NF-κB is protective against apoptosis in a number of experimental systems, we tested whether the inhibition of IkBα degradation by proteasome inhibitors was causally involved in cytoprotection. To specifically inactivate NF-κB, we infected SMC with an IRES-PURO retrovirus encoding a phosphorylation-resistant IκBα mutant (IκBα$^{Ser32,36/Ala}$) and the selectable puromycin resistance gene. This allowed rapid selection of a transduced SMC population expressing IκBα$^{Ser32,36/Ala}$ with approximately 99% infection efficiency as we have previously shown.[13,14] Transduced SMC containing the control vector were treated with collagen fragments in the presence or absence of LLnL, and SMC overexpressing IκBα$^{Ser32,36/Ala}$ were treated

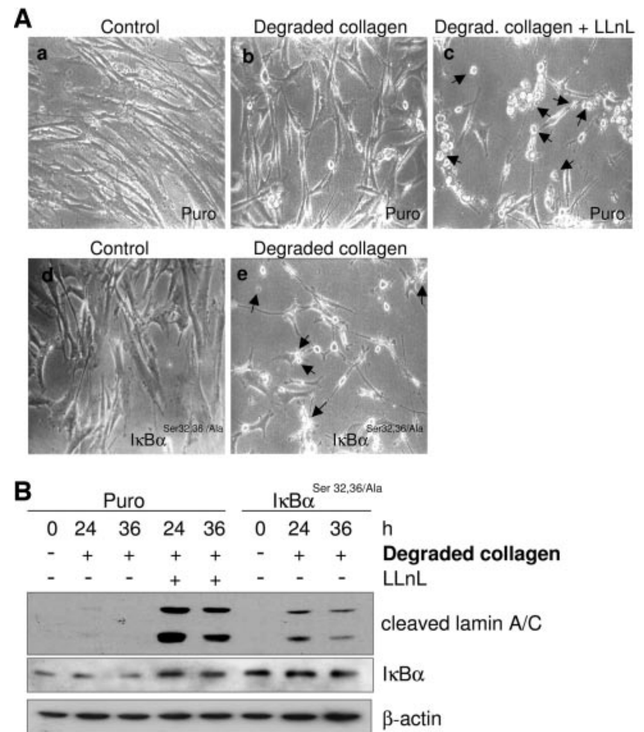

Figure 3. NF-κB inhibition by proteasomal inhibitors or overexpression of superrepressor IκBα$^{Ser32,36/Ala}$ induces apoptosis in SMC exposed to degraded collagen. A, Phase-contrast microscopy of SMC infected with pBMN-IRES-PURO retrovirus encoding a control vector (a through c) or IκBα$^{Ser32,36/Ala}$ (d and e) and treated with (b, c, and e) or without (a and d) degraded collagen for 24 hours. The morphology of vector-transduced SMC treated with degraded collagen and LLnL (50 μmol/L) is shown in c. Arrows indicate membrane blebbing as a morphological marker for apoptosis. B, SMC were treated as described in A and harvested after 24 and 36 hours of collagen fragment treatment, respectively. Apoptosis was evaluated by immunoblotting against cleaved lamin A/C. Total IκBα levels were determined by immunoblotting, and β-actin was used to confirm equal loading. Shown is a representative experiment of 3.

with collagen fragments alone (Figure 3). Although overexpression of IκBα$^{Ser32,36/Ala}$ per se did not induce any morphological changes, treatment with collagen fragments led to pronounced cell rounding, blebbing and detachment reminiscent of apoptosis (Figure 3A). Accordingly, Western blot analysis revealed distinct and specific cleavage of the caspase-6 substrate lamin A/C in SMC overexpressing IκBα$^{Ser32,36/Ala}$ after treatment with collagen fragments (Figure 3B). In contrast, SMC transduced with the control vector and treated with degraded collagen did not show lamin A/C cleavage unless LLnL was present (Figure 3B).

Blockade of $\alpha_v\beta_3$ Integrins Prevents NF-κB Activation and Leads to Apoptosis in the Presence of Degraded Collagen

To test whether degraded collagen activates NF-κB via binding to specific integrins, SMC were incubated with blocking antibodies against α_2, α_5 and $\alpha_v\beta_3$ integrins, respectively, and exposed to degraded collagen for 2 hours. Whereas blockade of α_2 and α_5 integrins had no effects on NF-κB DNA binding activity in EMSAs (data not shown), blocking antibodies to $\alpha_v\beta_3$ substantially inhibited NF-κB activation by degraded collagen (Figure 4A). Blockade of

Downloaded from http://ahajournals.org by on September 27, 2019

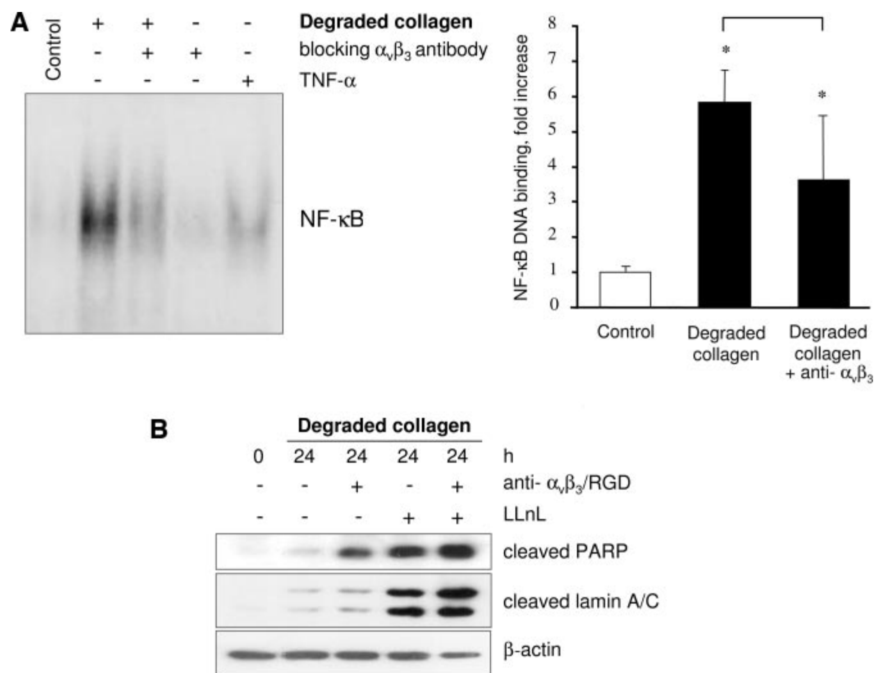

Figure 4. Blockade of $\alpha_v\beta_3$ integrins prevents NF-κB activation and leads to apoptosis in the presence of degraded collagen. A, Left, SMC were preincubated for 30 minutes with a blocking antibody against $\alpha_v\beta_3$ integrin (10 μg/mL per 500 000 cells) and exposed to degraded collagen for 2 hours. To exclude an effect on basal NF-κB activity, SMC were incubated with the antibody in the absence of degraded collagen for 2 hours. EMSA was performed as in Figure 2. Tumor necrosis factor-α (TNFα)-treated (50 ng/mL) SMC were used as a positive control. Shown is a representative experiment of 4. Right, NF-κB DNA binding activity was quantified densitometrically by NIH Image and presented as fold increase over untreated SMC. *$P<0.05$. B, SMC were incubated with 10 μg/mL blocking $\alpha_v\beta_3$ antibody and 100 μmol/L RGD peptides in the presence or absence of 50 μmol/L LLnL and exposed to collagen fragments for 24 hours. A nonblocking isotype-matched mouse IgG_1 was used as control. Apoptosis was evaluated by immunoblotting against PARP and cleaved lamin A/C. β-Actin was used as loading control. Shown is a representative experiment of 3.

$\alpha_v\beta_3$ in the absence of degraded collagen had no effect on basal NF-κB activity (Figure 4A). Furthermore, $\alpha_v\beta_3$ blockade by anti-$\alpha_v\beta_3$ antibodies and RGD peptides resulted in enhanced apoptosis as shown by PARP and lamin A/C cleavage and this was further enhanced by treatment with LLnL (Figure 4B).

Degraded Collagen Induces IAP Expression via Activation of NF-κB

NF-κB–dependent de novo gene expression is required for survival in many experimental systems,[16] and several members of the IAP family are under its transcriptional control, thereby mediating its antiapoptotic effects.[17,18] To determine whether IAPs may be involved in NF-κB–mediated protection in our system, we analyzed their expression in SMC exposed to degraded collagen. There was a rapid and potent induction of xIAP, c-IAP2, and survivin after 1 hour of collagen fragment treatment and their levels steadily increased up to 30 hours (Figure 5A). To test whether IAP induction was NF-κB-dependent, we retrovirally overexpressed the superrepressor $I\kappa B\alpha^{Ser32,36/Ala}$ and a control vector, respectively, and determined IAP levels after incubation of SMC with degraded collagen. The induction of xIAP and c-IAP2 by collagen fragments was substantially inhibited in $I\kappa B\alpha^{Ser32,36/Ala}$-expressing cells compared with control vector transduced cells (Figure 5B). In control SMC, LLnL inhibited the induction of xIAP and c-IAP2 by collagen fragments in a comparable manner (Figure 5B).

Inhibition of IAPs by siRNA Sensitizes SMC to Apoptosis in the Presence of Collagen Fragments

To test whether the induction of IAP expression in SMC after exposure to degraded collagen is necessary for survival, we knocked down IAP expression by siRNA. Initially, SMC were transfected with siRNA against xIAP, c-IAP2 and nonsilencing siRNA, respectively, and protein levels of the IAPs were determined by Western blot analysis. A marked reduction of xIAP and c-IAP2 protein expression was present 48 and 72 hours after siRNA treatment (Figure 6A), with no effect on basal apoptosis rate (data not shown). We then treated SMC with siRNA against xIAP, c-IAP2, or survivin and exposed them to degraded collagen for 18 hours. We observed that, in contrast to SMC treated with nonsilencing siRNA, apoptosis was distinctly induced in all IAP-siRNA–treated SMC, as shown by cleavage of lamin A/C, PARP and activation of caspase-3 (Figure 6B). These results suggest that the induction of IAPs is necessary and sufficient to protect SMC against apoptosis after treatment with degraded collagen.

Discussion

Type I Collagen Fragments Activate a Death and a Survival Pathway via Different Integrins: Functional Cross-Talk at the IAP Level

We have previously demonstrated that treatment of SMC with collagen fragments leads to a rapid activation of caspases and apoptosis via calpains.[11] However, as this process was moderate and the majority of the cells survived, we postulated the existence of a survival pathway activated in parallel by degraded collagen and dominating the calpain-mediated apoptotic pathway. In the present study, we identified this survival pathway as the robust activation of NF-κB via phosphorylation and degradation of IκBα. Its causal involvement was evident from the rapid submission of SMC to apoptosis when NF-κB activation was prevented by proteasome inhibitors or a superrepressor IκBα. We have previously shown that α_2 integrins mediate calpain activation by degraded collagen in our system[10] and have now identified $\alpha_v\beta_3$ integrins as responsible for NF-κB activation by the same collagen fragments. Blockade of $\alpha_v\beta_3$ not only prevented NF-κB activation but also resulted in apoptosis in the

Downloaded from http://ahajournals.org by on September 27, 2019

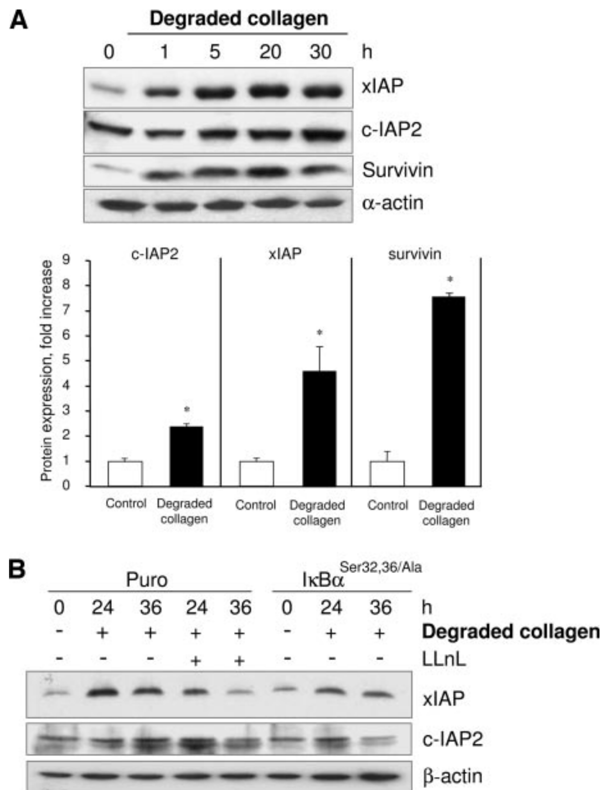

Figure 5. Degraded collagen fragments induce IAPs in an NF-κB–dependent manner. A, Whole cell extracts prepared from cells incubated with degraded collagen fragments for the indicated times were immunoblotted with antibodies against xIAP, c-IAP2, and survivin. α-Actin was used as loading control. Induction of IAP expression after 24 hours of treatment was quantified using NIH Image. Results are presented as mean±SD from 5 independent experiments. *$P<0.05$ vs control. B, SMC were infected with control PURO vector or $I\kappa B\alpha^{Ser32,36/Ala}$ and treated as described in Figure 2C. Protein levels of xIAP and c-IAP2 were determined by immunoblotting. β-Actin was used as loading control. Shown is a representative experiment of 3.

presence of collagen fragments, suggesting that $\alpha_v\beta_3$ integrins promote survival via induction of NF-κB in our system. Engagement of $\alpha_v\beta_3$ by ECM plays an important role in survival in several vascular cell types: (1) it protects endothelial cells against apoptosis after binding to osteopontin or fibronectin via NF-κB[19–21]; (2) its ligation by MMP-generated type I collagen fragments promotes survival in rat vascular SMC through tenascin-C[9,22,23]; and (3) MMPs secreted by human SMC cultured on polymerized collagen gels generate fragments capable of inducing NF-κB–dependent transcription.[13] Together, these data suggest that in our system, degraded collagen fragments simultaneously activated an apoptotic pathway triggered by α_2-mediated calpain/caspase activation and a survival pathway triggered by $\alpha_v\beta_3$-mediated NF-κB activation (Figure 7). The survival pathway was dominant over the apoptotic as substantial cell death took place only after inactivation of NF-κB. One possibility of a functional cross-talk between these 2 rival pathways may be at the level of the IAPs: whereas calpains proteolytically destroyed xIAP leading to loss of its caspase-inhibitory potential,[11] several IAPs including xIAP were potently induced by NF-κB. Such dynamic modulations of

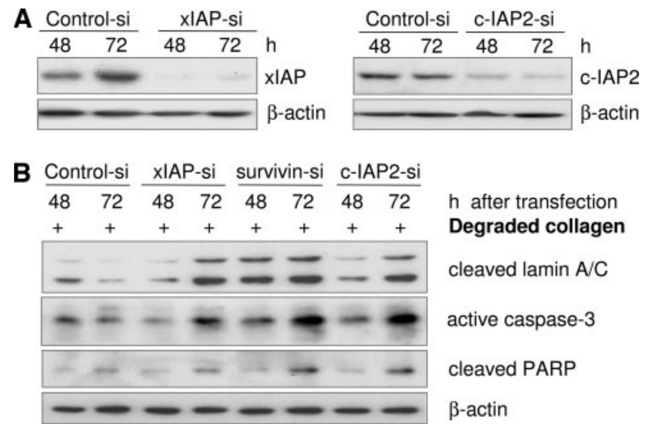

Figure 6. Collagen fragments induce apoptosis after siRNA treatment against IAPs. A, SMC were transfected with specific siRNA against xIAP, c-IAP2, or nonsilencing control siRNA, and IAP expression was analyzed by Western blotting 48 and 72 hours later. Shown is a representative experiment of 4. B, SMC were treated with siRNAs as in A and exposed to collagen fragments for 18 hours. Apoptosis was determined by immunoblotting with antibodies against cleaved lamin A/C, PARP, and active caspase-3. β-Actin was used as loading control. Shown is a representative experiment of 3.

IAP levels inside the cell may have a substantial impact on the activation threshold of caspases as autoprocessing and activation of caspases is known to occur in the absence of the IAPs.[24] Thus the functional dominance of the survival pathway over the apoptotic may be anchored in its ability to elevate the IAP threshold for caspase activation (Figure 7). An additional survival mechanism in our system may be mediated by Akt as collagen fragments activated Akt and were more effective in promoting apoptosis when Akt was inhibited (data not shown).

Our siRNA experiments have suggested that several IAPs were causally and independently responsible for survival as

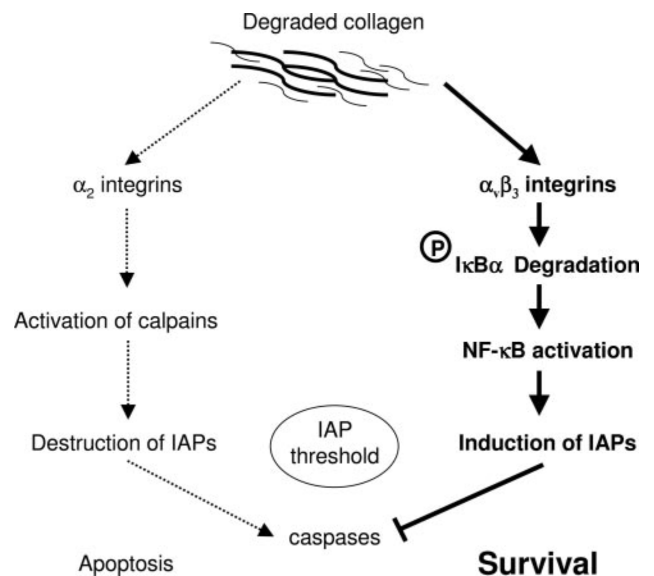

Figure 7. Schematic of a putative crosstalk between the calpain-mediated apoptotic pathway and the NF-κB–mediated survival pathway in SMC exposed to degraded collagen. The impact of both pathways on the IAP threshold for caspase activation may determine the sensitivity of SMC for apoptosis in the presence of degraded collagen.

Downloaded from http://ahajournals.org by on September 27, 2019

reducing the levels of single ones such as xIAP, c-IAP2, or survivin was sufficient to induce apoptosis. The inability to compensate or substitute for the antiapoptotic potential of one another suggests that individual IAPs play unique, nonredundant roles in our system. This is in line with the existence of several antiapoptotic functions beyond caspase inhibition of the individual IAPs such as the E3-ubiquitin ligase activity of xIAP toward active caspase-3 and -9,[25,26] and that of c-IAP2 toward caspase-7,[27] as well as the activation of the c-Jun N-terminal kinase-1 (JNK1) by xIAP but not c-IAP1 or c-IAP2, and its necessity for the antiapoptotic activity of xIAP.[28] In SMC, high-density cultures have been shown to exhibit high constitutive NF-κB activity and increased resistance to apoptosis attributable to enhanced expression of c-IAP1.[29] In endothelial cells, we and others have shown that IAPs such as xIAP were not only regulated by NF-κB but, vice versa, activated NF-κB themselves, thereby promoting their own transcription.[30,31] Thus, in our system, apart from directly inhibiting caspases, IAPs may be also enforcing survival through a positive feedback loop between their NF-κB–dependent transcription and their ability to, in turn, activate NF-κBs themselves.[30,31]

ECM Degradation in the Atherosclerotic Lesion: Pathways of SMC Death and Survival in a Proteolytic Environment

Apoptotic SMC are found in atherosclerotic lesions, and the low SMC numbers present in the thin fibrous caps of vulnerable plaques imply that SMC death may have occurred there over time. Eventually, such loss of SMC may potentially lead to decreased collagen synthesis in the fibrous cap and thus contribute to plaque destabilization and rupture.[32,33] Increased type I collagen proteolysis by activated MMPs is also considered a risk factor for plaque rupture[34] and has been shown to colocalize with MMPs in vulnerable plaques.[2,35,36] However, little is known about the particular type I collagen fragments generated in the atherosclerotic plaque in vivo: although the main type I collagen digesting enzymes such as MMP-1, -8, and -13 are abundantly present and active in the lesion, the only in vivo identified collagen fragments are those exposing the neoepitope generated after cleavage of the native triple-helical fibrils of type I, II, and III collagen at the collagenase-specific single site Gly775-Leu/Ile776.[35,36] According to well-established biochemical in vitro evidence, the resulting three-fourths and one-fourth collagen fragments would become accessible substrates to other MMPs present in the lesion such as gelatinases and stromelysins and be further catabolized.[37,38] Besides the MMPs, there is another class of collagen-degrading enzymes present in the atherosclerotic lesion, the cathepsins, and these are known to cleave type I collagen in vitro at sites that are different from those cleaved by the MMPs: cathepsins L and B cleave only telopeptides but not the native type I collagen triple helix, whereas cathepsin K cleaves both telopeptides at multiple sites within the triple helix.[39–41] Therefore, it is more than plausible that several different collagen fragments are present simultaneously and/or consecutively in the atherosclerotic plaque in vivo. The $\alpha_v\beta_3$ integrin is also present in lesions of atherosclerosis and appears to crucially influence the SMC content of the neointima after injury by regulating SMC migration.[42] As SMC degrade ECM during migration, this may be 1 possible scenario of a proteolytic environment where ECM fragments influence SMC survival by engaging $\alpha_v\beta_3$.

Macrophages and SMC are often neighbors in the atherosclerotic plaque, thereby actively interacting and influencing each other's biological behavior.[43,44] Because of this neighborhood, plaque SMC will most likely be confronted with matrix degradation, eg, by excessive production of MMPs by macrophages, at some point of their life. In a proteolytic environment, where protective outside-in signals from integrins are interrupted by the degradation of their ECM ligands, the existence of an antiapoptotic pathway originating precisely from some of the arising proteolytic ECM fragments may be important to guarantee SMC survival. Interestingly, apoptosis of SMC induced by mast cell chymase or lymphocyte-derived granzyme B in vitro have both been attributed to degradation of their fibronectin matrix and consecutive loss of protective focal adhesion signals.[45,46] In the much more complex microenvironment of atherosclerotic lesions, the presence of type I collagen fragments may counteract such apoptotic signals. Furthermore, high IAP levels maintained by collagen fragments may also protect SMC against other apoptotic stimuli present in the lesion such as reactive oxygen species, Fas, p53, and deadly cytokines.[5,6] In conclusion, our finding that collagen fragments tightly regulate SMC survival may be pathophysiologically essential not only at sites of increased ECM proteolysis such as atherosclerotic plaques but also in biological settings where ECM degradation is a general phenomenon necessary for cell migration, vascular remodeling, and neointima formation.[47,48]

Downloaded from http://ahajournals.org by on September 27, 2019

Acknowledgments

We thankfully acknowledge the technical assistance of Sina Mersmann, Kerstin Abouhamed, and Vanessa Brinkmann.

Sources of Funding

This study was supported in part by the Deutsche Forschungsgemeinschaft (LE 940/3-1), and the H.-H. Deichmann Foundation for Atherosclerosis Research. K.v.W.L. is a recipient of a postdoctoral fellowship from the Peter und Traudl Engelhorn Foundation.

Disclosures

None.

References

1. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ Res*. 2002;90: 251–262.
2. Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev*. 2005;85:1–31.
3. Barnes MJ, Farndale RW. Collagens and atherosclerosis. *Exp Gerontol*. 1999;34:513–525.
4. Ross R. Atherosclerosis–an inflammatory disease. *N Engl J Med*. 1999; 340:115–126.
5. Bennett MR. Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture. *Cardiovasc Res*. 1999; 41:361–368.
6. Kavurma MM, Bhindi R, Lowe HC, Chesterman C, Khachigian LM. Vessel wall apoptosis and atherosclerotic plaque instability. *J Thromb Haemost*. 2005;3:465–472.
7. Dhanabal M, Ramchandran R, Waterman MJ, Lu H, Knebelmann B, Segal M, Sukhatme VP. Endostatin induces endothelial cell apoptosis. *J Biol Chem*. 1999;274:11721–11726.

8. Maeshima Y, Yerramalla UL, Dhanabal M, Holthaus KA, Barbashov S, Kharbanda S, Reimer C, Manfredi M, Dickerson WM, Kalluri R. Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. *J Biol Chem*. 2001;276:31959–31968.
9. Jones PL, Crack J, Rabinovitch M. Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the alpha v beta 3 integrin to promote epidermal growth factor receptor phosphorylation and growth. *J Cell Biol*. 1997;139:279–293.
10. Carragher NO, Levkau B, Ross R, Raines EW. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J Cell Biol*. 1999;147:619–630.
11. von Wnuck Lipinski K, Keul P, Lucke S, Heusch G, Wohlschlaeger J, Baba HA, Levkau B. Degraded collagen induces calpain-mediated apoptosis and destruction of the X-chromosome-linked inhibitor of apoptosis (xIAP) in human vascular smooth muscle cells. *Cardiovasc Res*. 2006;69:697–705.
12. Levkau B, Scatena M, Giachelli CM, Ross R, Raines EW. Apoptosis overrides survival signals through a caspase-mediated dominant-negative NF-kappa B loop. *Nat Cell Biol*. 1999;1:227–233.
13. Ferri N, Garton KJ, Raines EW. An NF-kappaB-dependent transcriptional program is required for collagen remodeling by human smooth muscle cells. *J Biol Chem*. 2003;278:19757–19764.
14. Garton KJ, Ferri N, Raines EW. Efficient expression of exogenous genes in primary vascular cells using IRES-based retroviral vectors. *BioTechniques*. 2002;32:2–8.
15. Finco TS, Baldwin AS. Mechanistic aspects of NF-kappa B regulation: the emerging role of phosphorylation and proteolysis. *Immunity*. 1995;3:263–272.
16. Kucharczak J, Simmons MJ, Fan Y, Gelinas C. To be, or not to be: NF-kappaB is the answer–role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene*. 2003;22:8961–8982.
17. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med*. 1998;188:211–216.
18. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*. 1998;281:1680–1683.
19. Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM. NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. *J Cell Biol*. 1998;141:1083–1093.
20. Pritzker LB, Scatena M, Giachelli CM. The role of osteoprotegerin and tumor necrosis factor-related apoptosis-inducing ligand in human microvascular endothelial cell survival. *Mol Biol Cell*. 2004;15:2834–2841.
21. Scatena M, Giachelli C. The alpha(v)beta3 integrin, NF-kappaB, osteoprotegerin endothelial cell survival pathway. Potential role in angiogenesis. *Trends Cardiovasc Med.* 2002;12:83–88.
22. Jones PL, Jones FS, Zhou B, Rabinovitch M. Induction of vascular smooth muscle cell tenascin-C gene expression by denatured type I collagen is dependent upon a beta3 integrin-mediated mitogen-activated protein kinase pathway and a 122-base pair promoter element. *J Cell Sci*. 1999;112(pt 4):435–445.
23. Stringa E, Knauper V, Murphy G, Gavrilovic J. Collagen degradation and platelet-derived growth factor stimulate the migration of vascular smooth muscle cells. *J Cell Sci*. 2000;113(pt 11):2055–2064.
24. Salvesen GS, Dixit VM. Caspase activation: the induced-proximity model. *Proc Natl Acad Sci U S A*. 1999;96:10964–10967.
25. Suzuki Y, Nakabayashi Y, Takahashi R. Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc Natl Acad Sci U S A*. 2001;98:8662–8667.
26. Morizane Y, Honda R, Fukami K, Yasuda H. X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic Smac/DIABLO. *J Biochem (Tokyo)*. 2005;137:125–132.
27. Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J Biol Chem*. 2000;275:26661–26664.
28. Sanna MG, Duckett CS, Richter BW, Thompson CB, Ulevitch RJ. Selective activation of JNK1 is necessary for the anti-apoptotic activity of hILP. *Proc Natl Acad Sci U S A*. 1998;95:6015–6020.
29. Erl W, Hansson GK, de Martin R, Draude G, Weber KS, Weber C. Nuclear factor-kappa B regulates induction of apoptosis and inhibitor of apoptosis protein-1 expression in vascular smooth muscle cells. *Circ Res*. 1999;84:668–677.
30. Hofer-Warbinek R, Schmid JA, Stehlik C, Binder BR, Lipp J, de Martin R. Activation of NF-kappa B by XIAP, the X chromosome-linked inhibitor of apoptosis, in endothelial cells involves TAK1. *J Biol Chem*. 2000;275:22064–22068.
31. Levkau B, Garton KJ, Ferri N, Kloke K, Nofer JR, Baba HA, Raines EW, Breithardt G. xIAP induces cell-cycle arrest and activates nuclear factor-kappaB: new survival pathways disabled by caspase-mediated cleavage during apoptosis of human endothelial cells. *Circ Res*. 2001;88:282–290.
32. Kockx MM, Knaapen MW. The role of apoptosis in vascular disease. *J Pathol*. 2000;190:267–280.
33. Rekhter MD. Collagen synthesis in atherosclerosis: too much and not enough. *Cardiovasc Res*. 1999;41:376–384.
34. Katsuda S, Kaji T. Atherosclerosis and extracellular matrix. *J Atheroscler Thromb*. 2003;10:267–274.
35. 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.
36. Herman MP, Sukhova GK, Libby P, Gerdes N, Tang N, Horton DB, Kilbride M, Breitbart RE, Chun M, Schonbeck U. Expression of neutrophil collagenase (matrix metalloproteinase-8) in human atheroma: a novel collagenolytic pathway suggested by transcriptional profiling. *Circulation*. 2001;104:1899–1904.
37. Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J Clin Invest*. 1996;97:761–768.
38. Patterson ML, Atkinson SJ, Knauper V, Murphy G. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett*. 2001;503:158–162.
39. 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.
40. Liu J, Sukhova GK, Yang JT, Sun J, Ma L, Ren A, Xu WH, Fu H, Dolganov GM, Hu C, Libby P, Shi GP. Cathepsin L expression and regulation in human abdominal aortic aneurysm, atherosclerosis, and vascular cells. *Atherosclerosis*. 2006;184:302–311.
41. Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaisse JM. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. *J Biol Chem*. 1998;273:32347–32352.
42. Choi ET, Khan MF, Leidenfrost JE, Collins ET, Boc KP, Villa BR, Novack DV, Parks WC, Abendschein DR. Beta3-integrin mediates smooth muscle cell accumulation in neointima after carotid ligation in mice. *Circulation*. 2004;109:1564–1569.
43. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685–1695.
44. Lusis AJ. Atherosclerosis. *Nature*. 2000;407:233–241.
45. Choy JC, Hung VH, Hunter AL, Cheung PK, Motyka B, Goping IS, Sawchuk T, Bleackley RC, Podor TJ, McManus BM, Granville DJ. Granzyme B induces smooth muscle cell apoptosis in the absence of perforin: involvement of extracellular matrix degradation. *Arterioscler Thromb Vasc Biol*. 2004;24:2245–2250.
46. Leskinen MJ, Lindstedt KA, Wang Y, Kovanen PT. Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. *Arterioscler Thromb Vasc Biol*. 2003;23:238–243.
47. Newby AC, Zaltsman AB. Fibrous cap formation or destruction–the critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res*. 1999;41:345–360.
48. Littlewood TD, Bennett MR. Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol*. 2003;14:469–475.

Downloaded from http://ahajournals.org by on September 27, 2019