An NF-κB-dependent Transcriptional Program Is Required for Collagen Remodeling by Human Smooth Muscle Cells*

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Although remodeling of vessels can dramatically alter lumen diameter and clinical sequelae, the molecular mechanisms regulating extracellular matrix turnover and remodeling are still not well understood. To investigate these processes in human smooth muscle, we have compared their culture on monomer and polymerized collagen gels, conditions that mimic some of the features of injured and normal vessels, respectively. We show that culture on polymerized, but not monomer, collagen leads to the activation of the transcription factor NF-κB through phosphorylation and degradation of its inhibitor, IκBα. Coincident with NF-κB activation, expression of MMP1, MMP2, and α2 integrin increases on polymerized collagen. Specific inhibition of NF-κB by retroviral overexpression of wild-type IκBα or phosphorylation-resistant, IκBα-stabilized mutant (IκBαSer32,36/Ala) reverses the increases in MMP1 and α2β1 on polymerized collagen and decreases collagen gel contraction and degradation. However, forced overexpression of α2β1 integrin or MMP1 in smooth muscle cells expressing IκBαSer32,36/Ala rescues their ability to contract collagen gels. Thus, polymerized collagen induces NF-κB-dependent expression of MMP1 and α2β1 integrin, that are required for smooth muscle extracellular matrix remodeling.

Extracellular matrix (ECM) remodeling plays an important role in a wide variety of diseases and normal physiological processes such as arthritis, atherosclerosis, and wound healing. It is becoming clear that such remodeling involves dynamic interactions between resident cells and their matrix environment, but relatively little is known about the transcriptional programs by which ECM remodeling occurs. In vitro, fibroblasts cultured on free floating collagen gels can exert enough force to condense the matrix into dense organized structures thought to mimic some aspects of wound contraction (1). In this system, the transcription factor nuclear factor-κB (NF-κB), is activated, and one of its downstream target genes, α2 integrin, is required for gel contraction (2). Additionally, the expression of proteases involved in matrix degradation, including matrix metalloproteinase 1 (MMP1) and 3 (MMP 13), are also upregulated in fibroblasts cultured on polymerized collagen gels (3). These findings have suggested that the activation of specific transcription factors, and therefore transcriptional programs, likely play an important role in ECM remodeling. In support of this, it has been shown that a peptide inhibitor of NF-κB reduces induction of α2 integrin by polymerized collagen and the ability of fibroblasts to contract collagen gels (4).

ECM remodeling is thought to be an important step in the pathogenesis of several vascular diseases including atherosclerosis and post-angioplasty restenosis (5). In response to injury and to changes in blood pressure and flow, the ECM of the vessel wall can be extensively remodeled by vascular smooth muscle cells (SMCs). Similar to fibroblasts, adhesive events have been shown to regulate the activity of NF-κB in human SMCs in vitro. For example, adhesion of SMCs to fibronectin-coated dishes activates NF-κB (6), but serum and growth factors also induce NF-κB activation (7). Activated NF-κB is detected in intimal and medial SMCs in lesions of atherosclerosis, but not in healthy vessels (8). NF-κB immunostaining is also increased after balloon injury (9) and in coronary atherosclerotic plaques of patients with unstable angina (10). More significantly, blockade of NF-κB by adenoviral expression of its inhibitor, IκBα, after balloon dilation of the rabbit iliac artery reduces lumen narrowing as measured by angiography and morphometry (11). IκBα expression decreases macrophage recruitment and increases SMC apoptosis compared with control-treated contralateral vessels (11). Despite these in vivo effects, the role of NF-κB in ECM remodeling by vascular SMCs is not known.

In the current study, we have examined the effects of polymerized collagen gels on NF-κB activation in human aortic SMCs. Our experiments show that polymerized collagen, but not monomer collagen, specifically activates NF-κB through phosphorylation and degradation of the endogenous NF-κB inhibitor, IκBα. Retroviral overexpression of IκBα or a non-degradable phosphorylation-resistant IκBα-mutant significantly suppresses NF-κB activation and blocks collagen gel contraction by SMCs. In addition, we demonstrate that NF-κB activation is required for the induction of MMP1 and α2 integrin, which contribute to collagen degradation and remodeling. Overexpression of either of these two NF-κB gene products can rescue blockade of collagen gel contraction by IκBα overexpression, thus establishing a central role for NF-κB in vascular remodeling.

MATERIALS AND METHODS
Cell Culture and Collagen Matrix Preparation—Human newborn arterial SMCs were isolated from the thoracic aorta as previously...
described (12). SMCs between passages 5 and 9 were cultured in 1% PDS (plasma-derived serum)/DMEM (Dulbecco’s modified eagle’s medium) on the surface of the following collagen type I preparations: polymerized collagen gels (1.0 mg/ml final concentration) prepared by neutralization of the collagen solution (Vitrogen 100, Collagen Corporation) with one-sixth volume of 7X concentrated DMEM, dilution to a final 1X DMEM solution, and incubation at 37 °C for 24 h. Monomer collagen pellets were prepared by incubating 0.1 mg/ml collagen solution in 0.1 M acetic acid at 37 °C for 24 h and washed twice with DMEM before cell seeding. To alter cell adhesiveness to plastic, tissue culture dishes were coated with a concentration of poly (HEMA), or hydron powder (200 μl per well of a 12% solution in 95% ethanol, Interferon Sciences, Inc.) that was previously used to study the effect of cell shape on growth (13).

Inhibitors and Antibodies—26 S proteasome inhibitor N-p-tosyl-l-phenylalanine (TPCK) was purchased from Sigma Chemical Co. and the broad-based metalloproteinase inhibitor GM6001 from Elastin Products Co. The following antibodies were used: rabbit polyclonal anti-1αEA and phospho-1αEA (New England Bioslabs); rabbit polyclonal anti-p50 and anti-p65 (Santa Cruz Biotechnology); mouse monoclonal anti-MMP1 and anti-MMP2 (Calbiochem); mouse monoclonal anti-α2 integrin (P1H5 and P1H6) and mouse monoclonal anti-β1 integrin (P4C10) provided by W. G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA); rabbit polyclonal anti-α5 (Chemicon); rabbit polyclonal anti-β1 integrin (160) provided by R. O. Hynes (M. I. T., Cambridge, MA); mouse monoclonal anti-αv integrin (Transduction Laboratories); mouse monoclonal anti-α-actin (Sigma); and anti-CDS PF (phycoerythrin-conjugated) (BD Pharmedge).

Electrophoretic Mobility Shift and Luciferase Reporter Assays—Gel shift assays were performed as previously described (14). Cells cultured on monomer collagen were trypsinized, washed with phosphate buffered saline (PBS), and resuspended in 200 μl of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) and allowed to swell on ice for 20 min. SMCs cultured on polymerized collagen were incubated with 2.5 mg/ml of collagenase type I (Worthington Biochemical Corporation) for 15 min at 37 °C. The cell suspension was then washed with PBS and resuspended in buffer A as indicated above. The nuclei were separated from the cell lysates by centrifugation at 12,000 × g for 5 min at 4 °C in a microcentrifuge. The nuclear pellet was resuspended in 20 μl of buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.0, 25% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A) and incubated for 20 min on ice. The lysed nuclei were centrifuged for 5 min at 4 °C in a microcentrifuge. The nuclear extracts were assayed for protein content using the Bio-Rad BCA assay method. The binding reaction was carried out at room temperature for 15 min mixing 10 μg of nuclear extracts with 50,000 cpm of labeled probe, 4 μl of 5X binding buffer (50 mM Tris pH 7.5, 0.25 mM NaCl, 5 mM dithiothreitol, 5 mM EDTA pH 8.0, Ficoll Powder 1.5%, w/v) and 4 μg of poly(dIdC-dIdC) (to prevent nonspecific binding). The samples were run on 6% nondenaturing acrylamide gel. After running, the gels were dried, and bands were detected by autoradiography. For the supershift electrophoretic mobility shift assay (EMSA), the binding reactions were incubated with 1 μg of normal IgG or antibody recognizing NF-κB subunits (p65 and p50) for 15 min at room temperature before loading the sample on the gel.

For the luciferase reporter assay, SMCs were infected with pBMB-SIN-xB-Luciferase, cultured on monomer or polymerized collagen for indicated periods of time, and resuspended by either trypsinization or collagenase treatment, respectively. Luciferase activity was measured using a luciferase assay system (Promega) according to the manufacturer’s instruction.

Collagen Gel Contraction Assay and Collagen Degradation Assay—SMCs were incubated for 48 h in 1% PDS/DMEM, then trypsinized and seeded on the surface of polymerized collagen type I (1 × 10^5/35-mm Petri dishes). After 24 h the gel was detached from the bottom of the dish by the pipetting medium underneath the gel. Images were captured using Laboratory Imaging System (UVP) after 24, 48, and 72 h. The areas were calculated using NIH Image 1.59 software.

For the collagen degradation assay, SMCs were cultured on fluorescein isothiocyanate (FITC)-conjugated polymerized collagen (Elastin Products Co., 1.0 mg/ml final concentration), and conditioned media were collected at 24-h intervals and FITC-conjugated collagen fragments were measured using a Cyto Fluor Multi-Well Plate Reader (PerSeptive Biosystems).
Protein Analysis—Cells were washed twice with cold PBS and lysed in 100 μl of buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 10% glycerol, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 30 min on ice. Cell lysates were cleared by centrifugation at 15,000 × g for 20 min, and protein concentrations were determined using the BCA protein assay (Pierce). Lysates were separated by SDS-PAGE under reducing conditions, transferred to Immobilon polyvinylidene difluoride (Millipore) and subsequently immunoblotted with specific antibodies, prior to visualization by enhanced chemiluminescence (ECL, Amersham Biosciences). Quantitative densitometric analyses were performed using Lab Works Image Acquisition and analysis software (UVP, Inc.).

Flow Cytometry—Cell surface levels of α5 and β2 integrin were determined after dissolution of polymerized collagen by incubation with collagenase type I as described above. Cell suspensions were centrifuged at 1,000 rpm for 5 min, washed with PBS, and fixed with 4% paraformaldehyde. After fixation, cells were stained with mouse monoclonal antibodies, anti-β2 integrin (P1H5) followed by PE-conjugated secondary antibody. α5 and β2, integrin expression was measured by flow cytometry, using a FACScan™ (Becton Dickinson) and data analysis with CellQuest software.

Gelatin Zymography—Cell lysates were prepared in lysis buffer as described above, and conditioned media were collected from SMC cultures. Samples were prepared in nonreducing loading buffer and separated on 10% SDS-polyacrylamide gel containing 1 mg/ml of gelatin. After electrophoresis, gels were washed three times in washing solution (150 mM NaCl and 2.5% Triton X-100) for 30 min, rinsed in water, and incubated 12–16 h in collagenase buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM CaCl2). Gels were subsequently fixed and stained in Coomassie Blue fixative solution (25% methanol, 7% acetic acid, and 0.25% Coomassie Blue R250, Sigma) for 2 h at room temperature and destained with washing solution (25% methanol and 7% methanol in water) for 4–5 h.

Statistical Analyses—All data shown are representative of three replicate experiments unless otherwise noted. Data are presented as mean ± S.D. Student’s t test was used to evaluate differences between groups, and statistical significance was assigned when p < 0.05.

RESULTS

Polymerized Collagen Gel, but Not Monomer Collagen, Specifically Induces NF-κB Activation—We have previously shown that polymerized type I collagen can modulate SMC phenotype (17) and proliferation (18). To determine the effect of collagen on the activation of NF-κB in human SMCs, we compared SMCs cultured on monomer versus polymerized type I collagen. Total cellular levels of the p105/p50 NF-κB subunits appeared modestly reduced (77.5% ± 45.9 for p105 and 76.7% ± 50.9 for p50) on polymerized collagen as compared with monomer collagen, but the differences are not statistically significant in SMCs (Fig. 1A). However, polymerized collagen induces nuclear translocation (4.4 ± 1.6-fold) of the p50 NF-κB subunit (Fig. 1B). Although SMCs exhibit high constitutive NF-κB transcriptional activity (19), gel shift analysis shows that polymerized, but not monomer, collagen leads to approximately a 2.25 ± 0.52 increase (n = 4) in the DNA binding activity of NF-κB (Fig. 1C). NF-κB activation is observed as early as 1 h after plating SMCs on polymerized collagen and is sustained for 24 h (data not shown). Supershift analysis reveals that the slower migrating NF-κB complex consists of a p50/p65 heterodimer, whereas the faster migrating complex contains a p50/p50 homodimer, both of which are induced on polymerized collagen (Fig. 1C).

Activation of NF-κB-dependent transcription was confirmed using a reporter construct that shows a 3.7 ± 1.3-fold increase (n = 5 independent experiments) in expression of a NF-κB-dependent luciferase reporter gene when SMCs are cultured on...
polymerized collagen (Fig. 1D). Increased NF-κB-dependent transcription is observed as early as 4 h and sustained for at least 12 h after exposure to polymerized collagen (Fig. 1D). Similar results were observed with two different human SMC strains (data not shown). Although cell shape and focal adhesion formation are also altered on polymerized collagen (18), cell rounding and cytoskeletal disruption induced by cytochalasin D or colchicine does not lead to activation of NF-κB-dependent transcription (data not shown). Moreover, cell rounding induced by altering cell adhesiveness to plastic with (HEMA), a polymer originally used to examine the role of cell shape in growth control (13), induces only a 2.3-fold induction as compared with monomer (13), induces only a 2.3-fold induction (20, 21). Western blot analysis of SMCs plated on polymerized collagen demonstrates a rapid and sustained decrease (25% – 30% of the cells were not infected with IκBα-overexpressing retrovirus, as determined by flow cytometry analysis of the EGFP reporter (data not shown). As expected, the degradation-resistant IκBα0Ser32,36Ala mutant leads to a more pronounced inhibition of NF-κB DNA binding activity. The residual level of NF-κB activation is consistent with the fact that ~20–30% of the cells were not infected with the IκBα-overexpressing retrovirus, as determined by flow cytometry analysis of the EGFP reporter (data not shown).

To determine whether IκBα phosphorylation and degradation are required for NF-κB activation by polymerized collagen, SMCs were infected with an IRES-EGFP retrovirus encoding either wild-type IκBα, a phosphorylation-resistant IκBα mutant (IκBα0Ser32,36Ala) with increased stability (22), or an empty vector control. Western blot analysis of detergent cell extracts confirms that IκBα is expressed ~5–10-fold above endogenous levels after retroviral infection and is not significantly down-regulated in response to polymerized collagen (Fig. 2B). By gel shift analysis, overexpression of either IκBα or IκBα0Ser32,36Ala results in a marked inhibition of NF-κB DNA binding activity in cells plated on monomer or polymerized collagen (Fig. 2C).
NF-κB Transcriptional Activity Regulates Collagen Gel Contraction and Expression of α2β1 Integrin and Collagenase Type I in SMCs—SMC remodeling of the ECM can include contraction of the matrix through integrins, breakdown of the matrix by MMPs, and/or synthesis of new matrix (23). SMCs overexpressing IκBα or IκBαSer32,36Ala almost completely lose their ability to remodel floating collagen gels after 24–48 h, compared with control cells (Fig. 3, A and B). On polymerized collagen, human SMCs upregulate MMP1 (Fig. 4A) and activate MMP2 as early as 6 h after being plated (data not shown). Culture of SMCs on polymerized collagen for 24–48 h also increases expression of α2 integrin, by Western blot analysis (data not shown).

To test whether NF-κB transcriptional activity is required for the expression of MMPs and integrins in human SMCs plated on monomer versus polymerized collagen, we evaluated the effect of retroviral expression of IκBα or IκBαSer32,36Ala. Overexpression of either inhibitor markedly down-regulates MMP1 expression in SMCs plated on either monomer or polymerized collagen (Fig. 4A). Although polymerized collagen still induces expression of MMP1 in the conditioned media and cell lysates of SMCs overexpressing IκBαSer32,36Ala, the levels achieved are almost comparable to control EGFP transduced transduced cells plated on monomer collagen (81.3% ± 1.5 and 86.2% ± 3.9, respectively). Importantly, this response exactly mirrors the levels of NF-κB DNA binding activity seen after plating SMCs on monomer and polymerized collagen (Fig. 2C). In contrast, IκBα and IκBαSer32,36Ala overexpression does not inhibit either the activation or the expression of MMP2 by polymerized collagen (Fig. 4A and data not shown). IκBα or IκBαSer32,36Ala overexpression also decreases cell surface levels of α5 and β1 integrin (Fig. 4B), but does not alter the expression of α5 integrin or its down-regulation by polymerized collagen (data not shown).

MMP1 and α2 Integrin Expression Are Sufficient to Restore Collagen Gel Contraction in SMCs with NF-κB Blockade—To evaluate whether MMP and integrin activity are required for collagen gel contraction, we examined the effect of the broad spectrum metalloproteinase inhibitor GM6001 and blocking or non-blocking α2 integrin antibodies on collagen gel contraction by SMCs. The incubation of SMCs with 50 μM GM6001 leads to a 40% decrease in collagen gel contraction (Fig. 5A). In the presence of blocking (P1H6), but not of non-blocking (P1H5) α2 integrin antibodies, there is a dose-dependent inhibition of collagen gel contraction (Fig. 5B). Therefore, α2β1 integrin action at the cell surface as well as MMP activity are important for collagen contraction by SMCs. NF-κB involvement in collagen degradation was evaluated by plating SMCs on FITC-labeled collagen (Fig. 6A). Overexpression of IκBαSer32,36Ala significantly inhibits collagen release, while the broad-based MMP inhibitor GM6001 almost completely blocks degradation (Fig. 6A).

To test the possibility that MMP1 and α2β1 are downstream of NF-κB, we asked whether forced expression of MMP1 or α2 integrin could rescue the inhibition of collagen gel contraction induced by IκBαSer32,36Ala overexpression. SMCs infected with either IκBαSer32,36Ala-ires-EGFP or empty control vector were superinfected with an IRES-PURO retrovirus expressing either MMP1, α2 integrin, or empty control vector. Levels of α2 integrin were restored in IκBαSer32,36Ala-overexpressing cells to endogenous levels with retroviral infection with an α2 integrin-IRES-PURO retrovirus (Fig. 6B). Similarly, down-regulation of MMP1 by blockade of NF-κB was reversed by co-infection with an MMP1-expressing retrovirus (Fig. 6B). The overexpression of α2 integrin led to a significant increase in the level of MMP1, which was reduced by the simultaneous expression of IκBαSer32,36Ala (Figs. 6B and 7B). Overexpression of IκBαSer32,36Ala alone blocks collagen gel contraction, while co-expression of either MMP1 or α2 integrin efficiently restores the ability of SMCs to contract collagen gels (Fig. 6C). The overexpression of MMP1 or α2 integrin overexpression has no effect on gel contraction in control cells.

Rescue of Collagen Gel Contraction by α2 Integrin Requires MMP Activity—Since overexpression of α2 integrin increases the levels of MMP1 (Figs. 6B and 7B), its effects on collagen gel contraction may be mediated in part through MMPs. Consistent with this hypothesis, the MMP inhibitor GM6001 blocks the rescue of collagen gel contraction in IκBαSer32,36Ala-overexpressing cells co-transduced with α2 integrin (Fig. 7A). As previously observed, co-transduction of SMCs with IκBαSer32,36Ala and α2 integrin returns MMP1 levels to those in control cells, and the MMP1 levels are not altered by GM6001 treatment (Fig. 7B). In contrast MMP2 activation is independent of NF-κB activation and α2 integrin transduction, while...
Vitro data have correlated activation of NF-κB with the arterial response to injury (24), and activation of NF-κB contributes to lumen loss observed after balloon dilation (11, 25). Our studies have probed the NF-κB-mediated molecular mechanisms that regulate the remodeling response using collagen gel contraction; an accepted in vitro model of collagenous tissue remodeling.

Several studies have demonstrated a role for α5β1, α6β1, and αvβ3 integrins in mediating collagen gel contraction (2, 26, 27). Other classes of molecules have also been implicated in the process, such as MMPs (28), the membrane tetraspan protein CD9 (29), osteopontin (29), and α-smooth muscle actin (30). A limitation of all of these studies is a focus on the fibroblast and their reliance upon blocking anti-integrin antibodies and pharmacologic inhibitors of NF-κB, or transduced cell lines of uncertain physiologic relevance, or primary cells transduced at very low efficiencies. In contrast, in the present study we have utilized primary human SMCs transduced at high efficiencies with IRES-based retroviral vectors (15), and we demonstrate a central role for NF-κB and two of its gene targets in collagen gel contraction by SMCs.

Rigid as well as floating polymerized collagen gels induce NF-κB-dependent transcriptional activity of SMCs by 2–3-fold, while monomer collagen has no effect. These results contrast with those seen in cultured fibroblasts, where floating, but not attached polymerized collagen, induces NF-κB (4, 31). This discrepancy suggests that cell-type specific differences exist between fibroblasts and SMCs with regard to the activation of NF-κB by collagen. It is possible that the high constitutive NF-κB activity of SMCs, which is absent in fibroblasts (19),

**DISCUSSION**

*NF-κB Activation Induced by Polymerized Collagen Gels Is Required for Collagen Gel Contraction by Human SMCs—* Extracellular matrix remodeling after vascular injury is the result of a tightly regulated process that involves the synthesis, breakdown, and remodeling of collagen (23). Both in vivo and in vitro data have correlated activation of NF-κB with the arterial response to injury (24), and activation of NF-κB contributes to

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** Exogenous expression of either αv integrin or MMP1 can reverse the inhibitory effect of IκBαSer32,36/Ala on SMC-mediated collagen gel contraction. A, human SMCs were infected with pBM-IRES-PURO retrovirus encoding control vector or IκBαSer32,36/Ala and plated on FITC-conjugated polymerized collagen gels. The cells infected with control PURO vector were cultured in the presence or absence of the MMP inhibitor GM6001. Conditioned media were collected after 24, 48, and 72 h. Release of FITC-conjugated collagen was measured with a 96-well plate reader. The data are representative of duplicate experiments. B, after 24 h, polymerized collagen gels were released from the Petri dishes, and the final collagen areas were measured after 48 h with duplicate determinations. C, after 24 h, polymerized collagen gels were released from the Petri dishes, and the final collagen areas were measured after 48 h. The data are representative of two independent experiments with triplicate determinations.

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Rescue of collagen gel contraction by exogenous expression of αv integrin requires MMP activity. Human SMCs were infected with pBM-IRES-PURO retrovirus encoding control vector or IκBαSer32,36/Ala and then superinfected with pBM-IRES-PURO vector encoding either αv integrin or empty vector controls followed by plating on polymerized collagen gels, and the cells were cultured in the presence or absence of the MMP inhibitor GM6001 (50 μM). The data are representative of duplicate experiments. A, after 24 h, polymerized collagen gels were released from the Petri dishes, and the final collagen areas were measured after 48 h with duplicate determinations. B, after 72 h, total cell lysates were prepared and αv integrin, and MMP1 expression were evaluated by Western blot analysis and gelatin zymography.
NF-κB activation in SMCs is rapid and sustained in response to polymerized collagen, and contrasts with the rapid and transient activation of NF-κB induced by cytokines (7, 33). The prolonged activation of NF-κB in SMCs on polymerized collagen is observed in multiple strains of normal human SMCs, and is accompanied by a rapid and sustained decrease in the levels of IkBa. Phosphorylation and degradation of IkBa are required for NF-κB activation in response to polymerized collagen as demonstrated by the degree of inhibition observed after retroviral overexpression of wild-type IkBa or mutant IkBaSer32,36Ala (30 and 70%, respectively). This significant blockade of NF-κB activity prevents collagen gel contraction and reduces collagen degradation.

NF-κB and Regulation of α2 Integrin and MMP1—Among the molecules involved in ECM remodeling, integrins and MMPs are the best characterized (2, 23, 27). In our studies, blockade of NF-κB activation in SMCs by overexpression of IkBa or mutant IkBaSer32,36Ala inhibits the polymerized collagen-induced increase in the levels of both α2 integrin and MMP1, but has no effect on activation of MMP2. The increases in α2 integrin and MMP1 induced in SMC on polymerized collagen are dependent upon NF-κB activity. Both α2 integrin and MMP1 have previously been shown to be directly and/or indirectly regulated by NF-κB (4, 34, 35). Xu et al. (4) proposed an indirect regulatory mechanism in fibroblasts by which NF-κB directs the synthesis of another transcription factor that in turn activates the transcription of α2 integrin (4). In contrast, a NF-κB-like element in the MMP1 promoter has been identified (36), and α2β1 integrin expression has been shown to be required for the increased levels of MMP1 in fibroblasts on polymerized collagen (3). Consistent with the requirement for α2 integrin and NF-κB, we observed an up-regulation of MMP1 after overexpression of α2 integrin, and MMP1 is suppressed and dependent upon NF-κB activation in SMCs overexpressing IkBa or IkBaSer32,36Ala.

The overexpression of MMP1 in SMCs with blockade of NF-κB demonstrates that MMP1 is required and sufficient to rescue collagen gel contraction by SMCs. The exact extent of rescue of collagen gel contraction is difficult to assess since transduction levels in singly transduced cells is higher than in the double transduction studies. One limitation of the retroviral system is that the number of transduced cells is affected by inhibition of cell proliferation in SMCs overexpressing IkBaSer32,36Ala (data not shown). By FACS analysis, we observe ~40–45% of cells expressing IkBaSer32,36Ala in double transduction experiments versus 70–75% after single infection. However, these studies clearly establish the sufficiency of MMP1 expression to partially reverse the blockade of NF-κB activation.

Overexpression of α2 integrin is also sufficient to restore collagen gel contraction in SMCs with blockade of NF-κB activation. However, the rescue of collagen gel contraction by α2 integrin overexpression is dependent upon MMP activity as the MMP inhibitor GM6001 completely blocks rescue. As previously described (3), we observed higher MMP1 levels in SMCs overexpressing α2 integrin, suggesting an indirect rescue mechanism of collagen gel contraction by α2 integrin. Although the nature of the MMP dependence is unclear, it is possible that tensile forces exerted by SMCs through α2 integrin-dependent anchorage to collagen can be enough to condense the matrix into dense organized structures only when coordinated with collagen breakdown. In addition, MMP1 has also been shown to bind to α2β1 integrin in keratinocytes on native type I collagen (37). The increase in α2β1 integrin could therefore promote localized proteolytic events to the site of substrate, thereby promoting collagen remodeling.

Implications for Vascular Remodeling—Our results suggest that there may be two distinct pathways regulating NF-κB-dependent remodeling: one that requires α2 integrin and presumably “sensing” of tensional forces, and the other that requires collagen breakdown by MMP1 (Fig. 8). However, both pathways are dependent upon proteolytic activity since the rescue of NF-κB blockade by α2 integrin is blocked by the broad-based MMP inhibitor GM6001. This inhibitor blocks MMPs and the related disintegrin and metalloproteinase (ADAM) family of proteases, and our data did not allow us to identify the specific protease(s) required.

The recent application of adenovirus carrying human IkBa after balloon dilation of the rabbit iliac artery demonstrated that efficient transduction of medial SMCs as well as adventitial cells is sufficient to reduce lumen narrowing with a lumen gain of ~40% (11). Although MMP1 and α2 integrin (or α1 integrin) levels were not evaluated in these animals, we would predict that their expected reduced expression could contribute to the lumen gain.

The transduction with IkBa into the rabbit vessels did not significantly influence cell proliferation at the time points evaluated, but a 3-fold increase in the number of apoptotic medial SMCs was observed 5 days after angioplasty that may be partially due to reduction in NF-κB-induced inhibitors of apoptosis proteins (11). Transduction with IkBa also significantly reduced the adhesion molecule ICAM-1 and the monocyte chemoattractant protein-1 (MCP-1) that are likely to have been involved in the 90% reduction in macrophage recruitment. The pleiotrophic effects of blockade of NF-κB in the artery wall make it difficult to evaluate the individual contribution of each of the changes. However, the data clearly demonstrate that activation of NF-κB contributes to lumen loss following balloon...
angioplasty. Our dissection of two NF-κB-specific gene products, MMP1 and α5 integrin, as key mediators of SMC collagen gel contraction raise the possibility of their contribution to the observed changes in vessel remodeling.

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