Research Article

NF-κB and Matrix-Dependent Regulation of Osteopontin Promoter Activity in Allylamine-Activated Vascular Smooth Muscle Cells

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Repeated cycles of oxidative injury by allylamine in vivo induce a proliferative rat vascular (aortic) smooth muscle cell (vSMC) phenotype characterized by matrix-dependent enhancement of mitogenic sensitivity, changes in cell surface integrin expression, and osteopontin (opn) overexpression. Here, we show that constitutive and mitogen-stimulated NF-κB DNA binding activity is enhanced in allylamine vSMCs. Matrix-specific changes in cellular Rel protein expression were observed in allylamine vSMCs. The NF-κB DNA binding element located at −1943 in the 5′-UTR strongly inhibited opn promoter activity in allylamine vSMCs, and this response was regulated by the extracellular matrix. Constitutive increases in opn promoter activity were only seen when allylamine cells were seeded on a fibronectin substrate, and this response was independent of the NF-κB DNA binding sequence within the regulatory region. Thus, NF-κB functions as a critical regulator of the allylamine-induced proliferative phenotype in vSMCs.

1. Introduction

Atherosclerosis is a chronic inflammatory disease associated with thickening of the arterial wall and reduction in vessel lumen size. The atherosclerotic lesion consists of a mixture of immune cells, extracellular matrix, fatty deposits, and phenotypically modified vSMCs [1]. Injury of vSMCs produces atherogenic (i.e., activated) phenotypes characterized by increased proliferative and migratory activities and loss of differentiation [2]. These characteristics are replicated when blood vessels are subjected to viral, chemical, or physical injury in vivo [3–5], or when vSMCs are cultured in the presence of mitogens [6], or seeded at low densities on a growth-permissive fibronectin matrix [7]. The nature and extent of phenotypic modulation is subject to regulation since variable degrees of phenotypic drift are seen depending upon the nature of the stimulus and the culture conditions in vitro [8]. The finding that vSMCs isolated from atherosclerotic vessels express characteristics of the activated phenotype suggests that the atherosclerotic process involves reprogramming of vascular gene expression. This is comparable to the changes seen in vSMCs isolated from animals challenged chronically with allylamine (CH₂=CHCH₂NH₂), a primary amine metabolized within the vascular compartment to acrolein and hydrogen peroxide [9].

Repeated exposure of rats to allylamine induces vascular lesions of atherosclerotic morphology that arise from the injury caused by acrolein and hydrogen peroxide, byproducts of oxidative deamination within the vascular wall [10]. vSMCs are enriched in a copper-containing amine oxidase that catalyzes the metabolism of allylamine and mediates depletion of cellular glutathione, direct oxidant injury to the tunica media, and phenotypic modulation of vSMCs to less differentiated phenotypes. In vitro analysis of vSMCs isolated from allylamine-treated animals revealed disorganization of the contractile apparatus, increases in endoplasmic reticulum, and emergence of a prominent nucleolus [11]. These activated vSMCs acquire a significant proliferative advantage
over vSMCs from control counterparts and exhibit enhanced phosphoinositide-3-kinase (PI-3-K) activity, secretion, and deposition of matrix proteins, and modulation of integrin-coupled NF-κB signaling [12].

NF-κB is a dimeric transcription factor that resides in the cytoplasm in association with an inhibitory protein, IκB. The dimer consists of members of the Rel protein family including RelA/p65, RelB, c-Rel, p50, and p52. In response to a positive stimulus, IκB is phosphorylated by Iκ-κ-kinase (IKK). Phosphorylation of IκB renders the protein susceptible to ubiquitination and proteolysis, allowing the NF-κB dimer to translocate to the nucleus and bind cis-acting regulatory sequences [13]. The ability of NF-κB to activate or repress target genes also depends on the phosphorylation status of Rel proteins [14]. NF-κB dimers of varying composition bind preferentially to distinct DNA sequences thus providing another point for differential regulation [15]. NF-κB regulates genes involved in the inflammatory, mitogenic, and apoptotic response. Of interest is opn, a cytokine with prominent roles in immunity, bone remodeling, cancer, multiple sclerosis, arthritis, kidney dysfunction, and atherosclerosis. opn is sequestered in the extracellular matrix of atherosclerotic plaques and binds to αcβ3, αcβ4, αvβ3, and αvβ1 integrin receptors on vSMCs [16, 17] to modulate proliferation, migration, and expression of inflammatory cytokines. The opn gene is regulated by upstream stimulatory factor 1 (USF1) and activator protein-1 (AP-1) transcription factors [18], but to date a definitive role for NF-κB has not been described. Detailed correlations between opn and atherosclerosis in humans [19, 20], and in animal models, have been reported [21].

We have previously described a central role for integrin-extracellular matrix signaling in vSMC proliferation [12]. Evidence is presented here that extracellular matrix- and DNA sequence-specific changes in NF-κB activity regulate opn promoter activity. These data indicate that NF-κB participates in the regulation of vSMC phenotypes by oxidative chemical injury.

### 2. Materials and Methods

#### 2.1. Materials

Allylamine (99% pure) was purchased from Aldrich (Milwaukee, WI). Medium 199, trypsin, and antibiotic/antimycotic solution were purchased from Gibco (Grand Island, NY). [32P]-ATP was purchased from NEN Radiochemicals (Grand Island, NY). NF-κB consensus oligonucleotide and T4 polynucleotide kinase were purchased from Promega (Madison, WI). Antibodies for Rel proteins, IκBα, IκBβ, and IKK were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). The antibody for phosphoserine was obtained from Zymed Laboratories Inc. (San Francisco, CA). Protein A-sepharose beads were obtained from Amersham Biosciences (Piscataway, NJ). Matrix-coated plates (BioCoat) were purchased from Becton Dickinson (Franklin Lakes, NJ). Nitrocellulose membranes were purchased from BioRad (Hercules, CA), Dura chemiluminescent substrate was purchased from Pierce (Rockford, IL). Kits for plasmid and RNA purification and DNase treatment were from Qiagen (Valencia, CA). The QuikChange Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA). Fugene 6 was from Roche (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

#### 2.2. Cell Culture

vSMCs were isolated by enzymatic digestion of aortas from 12 adult male Sprague-Dawley rats gavaged with allylamine (70 mg/kg) or 12 control rats gavaged with tap water for 20 days as described in detail previously [8]. Subcultures were prepared by trypsinization of primary cultures and maintained in Medium 199 supplemented with 10% FBS and 2 mmol/L L-glutamine in 5% CO2:95% air at 37°C. G0 synchronization was accomplished by incubation of cell cultures for 72 hours in Medium 199 containing 0.1% FBS as described.

#### 2.3. Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared using standard methodologies, quick frozen in liquid nitrogen, and stored at −80°C. For EMSA, 5 μg of nuclear protein was incubated with [32P]-end-labeled NF-κB consensus oligonucleotide for 1 hour. End labeling of oligonucleotides was performed with T4 polynucleotide kinase and γ[32P] ATP. Binding reactions were performed in 0.25x HEGDK (25 mmol/L HEPES pH 7.6, 1.5 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, 0.5 mol/L KCl) buffer supplemented with 1 mmol/L DTT, 20 μg BSA, and 50 ng poly-dIdC. After addition of loading dyes, reactions were electrophoresed on 7% nondenaturing polyacrylamide gels at 25 mA in 0.5x TBE (0.045 mol/L Tris, 0.045 mol/L Boric acid, 0.001 mol/L EDTA). Gels were dried and exposed to Kodak X-OMAT film with an intensifying screen for autoradiography at −80°C for 48 hours.

#### 2.4. SDS-PAGE and Western Blotting

Subconfluent cultures were rinsed with PBS and scraped. The pellet was resuspended with RIPA buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% NP-40, 10% sodium deoxycholate, 1 mg/mL leupeptin, 1 mg/mL aprotinin, 1 mg/mL pepstatin) and stored at −20°C. Three-10 μg of protein were diluted in sample buffer, boiled for 5 minutes, and loaded into a 7% denaturing polyacrylamide gel. Electrophoresis proceeded at 18–22 mA until tracking dyes reached the bottom. The gels were equilibrated in transfer buffer for 5 minutes and then transferred to nitrocellulose membranes for 9 hours at 20 V at 4°C. Membranes were blocked in 5% milk in TBS (0.1 mol/L Tris pH 7.5, 0.15 mol/L NaCl) with 0.1% Tween (TTBS) for 18 hours then probed overnight with a 1:1000 dilution of rabbit primary antibody. After 6 × 5 min washes in TTBS, membranes were incubated for 4 hours with a 1:25000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP). Following another series of washes, membranes were incubated for 5 minutes in a 1:2 dilution of chemiluminescent substrate and then visualized for 30 seconds to 2 minutes on Kodak X-OMAT film.

#### 2.5. Immunoprecipitation

Twenty five μg of protein was incubated overnight at 4°C with 1 μg of antibody against...
IKK. Fifty μL of a 50% slurry in TE (10 mmol/L Tris, 1 mmol/L EDTA pH 8.0) of protein A-sepharose was added to each immunoprecipitation reaction, and agitated for 4 hours at room temperature. The sepharose beads were pelleted and washed three times with TBS. Pellets were resuspended in Laemmli sample buffer and boiled for 5 minutes. After sepharose, beads were pelleted, 10 μL of supernatant was loaded for SDS-PAGE.

2.6. Synthetic Oligonucleotides. Following examination of published sequences for mouse c-Ha-ras promoter, rat opn promoter, and rat matrix metalloproteinase-9, oligonucleotides corresponding to NF-κB binding domains in these promoters were synthesized. The Genbank accession numbers and sequences used for each are shown in Table 1.

2.7. Generation of opn Promoter Constructs. The full-length rat opn promoter was provided by Dr. A. Ridall (UT Health Science Center, Houston, TX). 2094 base pairs were ligated into pGL2-basic using Nhel and Xhol restrictions sites. This construct was dubbed 2094 rOPNpr and sequential deletions generated beginning at 1157 (1157 rOPNpr), 672 (672 rOPNpr), and 127 (127 rOPNpr), respectively. MatInspector v2.2 (available at http://www.generegulation.de/) identified a proliferative vSMC phenotype [11, 12]. To evaluate the role of NF-κB in the regulation of the proliferative alaylamine phenotype, experiments were conducted to examine a time course of NF-κB binding activity to a consensus sequence in quiescent vSMC subcultures following mitogenic stimulation. Cells were synchronized by serum restriction and stimulated into growth with 10% serum for up to 5 hours. The profile of NF-κB binding activity in allylamine-modified vSMCs was different from control counterparts, with 3 distinct complexes detected at different times (Figure 2(a)). Constitutive NF-κB binding activity was enhanced in allylamine-modified vSMCs relative to controls. Mitogenic stimulation induced DNA binding activity as early as 0.5 hr in both cell types, but the predominance of individual complexes over time was phenotype-specific. Increased DNA binding activity was observed at all times in allylamine cells.

Because nuclear translocation of NF-κB is known to be dependent upon the degradation of IκB proteins [13], experiments were conducted next to evaluate steady-state levels of IκBα and IκBβ across the full synchronization and cell cycle transition regimen. The relative abundance of IκBα was not affected by growth factor deprivation since comparable protein levels were detected in synchronized cultures, as well as 0.5 hours after mitogen addition (Figure 2(b)). Degradation of IκBα was observed in the activated vSMC phenotype within 1 hr of serum stimulation, a response that peaked by 2 hr and began to subside by 3 hr. Modest reductions in IκBα were observed in control vSMCs, showing that early activation of NF-κB in response to mitogenic stimulation is a characteristic feature of the allylamine phenotype (Figure 2(b)). No degradation of IκBβ was observed in either cell type at any of the time points studied.

2.8. Transfections. vSMCs were seeded at a density of 4 × 10^4 cells per well in a 12-well plate and allowed to recover for 8 h before transfection with luciferase constructs and a β-gal-gal-expressing plasmid at a ratio of 1.5 : 1.0 μg. Plasmids were mixed at a ratio of 6 μL to 1 μg DNA in serum-free Medium 199 and added to the cultures for 36 h. Wells were rinsed with PBS and harvested using a lysis buffer included in Tropix’s Galacto-Light Reporter Assay Kit (Foster City, CA). Lysates were frozen until analysis. Twenty-five μL of lysate was pipetted into two 96-well plates and either combined with 80 μL of a luciferin working solution to measure luminescence in a BMG’s Fluostar OPTIMA plate reader, or 70 μL of Tropix’s β-gal reagent. β-gal samples were incubated for 1 hr before the addition of 100 μL of accelerator solution for measurement using an OPTIMA plate reader.

2.9. Statistics. All other statistical relationships were examined by ANOVA and Fisher’s least significant difference, unless otherwise noted. Statistical significance was assigned at the P < 0.05 level.

3. Results

Repeated cycles of oxidative injury by allylamine induce a proliferative vSMC phenotype [11, 12]. To evaluate the

| Table 1 |
| --- |
| **Mouse H-ras** |  |
| (AF268311) |  |
| 5'GGG GGT GGG TCT CTC TTT 3' |
| **Rat opn** |  |
| (AF017274) |  |
| 5'GAT TTT GGG AAT TTC CCT GCA 3' |
| **Rat MMP-9** |  |
| (AJ428366) |  |
| 3'CCC CAA TCC CCC AAC GGG CAC 5' |
To evaluate the possibility that ECM-dependent

patterns were observed in 3 separate experiments. The results show average number of cells in 5–7 replicate dishes during primary
culture or at passages 3 and 25 for the two cell types. Similar
expression was elevated in allylamine cells seeded on plastic or collagen I but decreased slightly on fibronectin. In contrast, p50
expression was elevated in allylamine cells seeded on plastic or collagen I but remained unchanged when cells were seeded
on fibronectin.

Phenotype-specific changes in the prevalence of Rel proteins may translate into variable composition of NF-κB dimers. Such alterations may influence the profile of tran-
scriptional regulation since different dimers may prefer-
entially recognize different target sequences. To test this
concept, NF-κB binding elements from four different pro-
moters were used to evaluate binding activity in nuclear
extracts from control and allylamine vSMCs seeded on
plastic. Up to four major binding protein complexes could
be identified, two of which were conserved across all of
the NF-κB binding sequences examined (Figure 4). With
the exception of proteins bound to the NF-κB sequence from the rat matrix metalloproteinase-9 (MMP-9) promoter, band
intensities were higher in nuclear extracts from allylamine-
activated vSMCs compared to controls, indicating a higher
degree of NF-κB activation. The intensity of the two faster-
mobility complexes (C3 and C4) was sequence specific, with
strong binding observed to the NF-κB consensus and c-Ha-
ras sequences.

Enhanced expression of opn mRNA and protein char-
terizes the allylamine phenotype[23]. Because increased
NF-κB binding to the opn promoter may contribute to
differential gene regulation, experiments were conducted
next to evaluate opn promoter activity in control and ally-
lamine vSMCs. A schematic for serial deletions is shown in
Figure 5(a). Activation of luciferase reporter constructs was
observed in both cell types, with considerably higher activ-
ities seen upon progressive deletion of upstream sequences
down to 572 bp (Figure 5(b)). Thus, inhibitory sequences
located between (2094 and 1157) and (1157 and 672) appear

| Binding site | Sequence |
|-------------|----------|
| VDRE        | Original: 5‘ CTTGGAGGTCTCATATGCCTCAGCTCCGAGG 3’ |
|             | Mutated: 5‘ CCTGGAAGGTCTCATATGCCTCAGCTCCGAGG 3’ |
| AP-1        | Original: 5‘ TCGTGTCTAGCTTCCGTGAGGC 3’ |
|             | Mutated: 5‘ TCGTGTCTATTCCTCCGTGAGGC 3’ |
| NF-κB       | Original: 5‘ AGGATTCTGGAAATTTCCCTGCACAGC 3’ |
|             | Mutated: 5‘ AGGATTCTGGAAATTTCCCTGCACAGC 3’ |
| USF1        | Original: 5‘ AAACAAAATCTATGTTGGTTGCTGCTTT 3’ |
|             | Mutated: 5‘ AAACAAAATCTATGTTGGTTGCTGCTTT 3’ |

Transcription factor binding sites were mutated using Stratagene’s QuickChange and Site-Directed Mutagenesis Kit. Binding sites are underlined, and mutated bases shown in lowercase.

Figure 1: Stability of the allylamine-activated vascular smooth muscle cell phenotype during serial propagation in vitro. Vascular smooth muscle cells were isolated from control and allylamine-treated rats and established in primary culture or passaged for up to 25 passages using established procedures. Cells were seeded at a density of 150 cells/mm² and incubated with 10% fetal bovine serum for four days in culture. The results shown represent the average number of cells in 5–7 replicate dishes during primary culture or at passages 3 and 25 for the two cell types. Similar patterns were observed in 3 separate experiments. The results show that allylamine cells exhibited a proliferative advantage that was sustained for multiple passages in vitro.

Table 2: Site-directed mutagenesis of opn promoter constructs.
Figure 2: Activation of NF-κB signaling in allylamine cells. Panel (a) shows NF-κB binding activity in control and allylamine cells over the course of a synchronization and mitogenic stimulation cycle. Cells were seeded at equivalent densities on plastic tissue culture dishes and nuclear extracts collected at multiple time points as noted. EMSA were performed using end-labeled NF-κB consensus oligonucleotide as a probe. Arrows denote the major NF-κB binding complexes identified. Similar results were seen in 2 independent experiments. C # denotes individual complexes. Panel (b) shows IκBα and IκBβ protein levels in control and allylamine cells over the course of a synchronization and mitogenic stimulation cycle. Cells were seeded at equivalent densities and serum restricted for 72 hr to synchronize in G0, and then released into growth by the addition of 10% fetal bovine serum. Crude protein extracts were harvested at various times after addition of complete medium. Protein extracts were electrophoresed, electroblotted onto nitrocellulose, and probed for each IκB. Similar results were observed in 3 separate experiments. C: control; A: allylamine. Panel (c) shows serine phosphorylation levels of immunoprecipitated IKKα and IKKβ in control and allylamine cells. Immunoprecipitated IKKα/β was electrophoresed and transferred onto two nitrocellulose membranes and blocked overnight in 5% milk. One membrane was probed for IKKα/β, and the other for phosphoserine, followed by incubation with horseradish peroxidase- (HRP-) labeled secondary. Membranes were then incubated with chemiluminescent substrate and visualized using the KODAK Image Station. Densitometry was performed using Kodak 1D Image Software. □: control, ■: allylamine.

Examination of sequences between −2094 and −1157 revealed putative cis-acting elements for AP-1, NF-κB and USF, as well as the well-characterized VDRE. To evaluate the relative contribution of these elements, the inducibility of mutant promoter constructs generated by site-directed mutagenesis was examined (Figure 5(c)). In control cells, disruption of any one of the elements significantly reduced luciferase activity (Figure 5(d)), implicating these elements in positive regulation of opn promoter activity. Interestingly, in allylamine cells only mutation of the VDRE reduced promoter activity, while mutation of AP-1 was inert, and mutation of NF-κB and USF binding sites returned transcriptional activity to full-length control levels. These results suggest that the opn promoter is under negative regulation by NF-κB and USF.

Lastly, the influence of a collagen I or fibronectin matrix on opn promoter activity was examined. The pattern of opn promoter activity was influenced by the matrix on which the cells were seeded with no changes seen between allylamine and control cells when cells were seeded on collagen (a nonpermissive matrix), and marked increases in allylamine cells over control cells when cells were seeded on fibronectin (a proliferation-permissive matrix), Figure 6(a). Deletion of the NF-κB binding site slightly decreased promoter activity in control cells but increased promoter activity in allylamine cells seeded on collagen I. The positive regulatory influence of fibronectin on opn promoter activity was not influenced by mutation of the NF-κB binding site (Figure 6(b)).

4. Discussion

Allylamine injury induces vascular lesions in the rat aorta that resemble those seen in atherosclerotic vessels [24]. This response involves accumulation of oxidative byproducts to significantly repress promoter activity in control and allylamine vSMCs. Promoter activity was completely lost in the minimal −127 promoter construct, and in all instances, promoter activity was considerably lower in allylamine cells compared to controls.
NF-κB vessels may involve alterations in NF-κB control contributes to phenotypic transition. Genomic

The activated aortic vSMC phenotype induced by allylamine − oligo (Promega). Gels were dried and exposed to film for 48 hours

promoters (Ha-ras, rOPN, and rMMP-9), as well as a consensus

subjected to EMSA against NF-κB to a panel of NF-κB binding complexes identified. C: control; a: allylamine. Similar results were seen in three separate experiments. C: control; A: allylamine.

Figure 3: Expression of Rel proteins in control and allylamine cells seeded on plastic, collagen I, fibronectin, and laminin. Protein extracts from cells at 80% confluence were electrophoresed in a 7% denaturing polyacrylamide gel, transferred onto nitrocellulose, and blocked overnight in 5% milk in TBS with 0.1% Tween. Membranes were probed overnight followed by incubation with horseradish peroxidase-labeled secondary antibody for 4 hours and then incubated with 1:2 chemiluminescent substrate: TTBS for 5 minutes, followed by visualization through autoradiography. Similar results were seen in three separate experiments. C: control; A: allylamine.

Figure 4: DNA binding activity in control and allylamine vSMCs to a panel of NFκB binding sequences. Nuclear extracts were subjected to EMSA against NF-κB binding domains from several promoters (Ha-ras, rOPN, and rMMP-9), as well as a consensus oligo (Promega). Gels were dried and exposed to film for 48 hours at ~80°C with intensifying screen. Arrows denote major NF-κB binding complexes identified. C: control; a: allylamine. Similar results were seen in multiple experiments.

within the vascular compartment, depletion of cellular glutathione, and activation of redox signaling [10, 25, 26]. The activated aortic vSMC phenotype induced by allylamine is stable suggesting that reprogramming of proliferative control contributes to phenotypic transition. Genomic reprogramming in vSMCs isolated from allylamine-injured vessels may involve alterations in NF-κB signaling since NF-κB DNA protein binding is markedly enhanced. These

findings parallel the molecular alterations seen in vSMCs isolated from atherosclerotic lesions [27, 28], and during the chronic hyperproliferative response to inflammation [28]. The present studies detail the molecular basis of NF-κB dysregulation in allylamine vSMCs.

Disruption and remodeling of the vessel wall matrix is a pivotal process in atherogenesis where expression of activated vSMC phenotypes correlates with disruption of the basal lamina by macrophage metalloproteinases [29]. As the process of phenotypic modulation progresses, activated vSMCs change the relative composition of the extracellular matrix [30]. Collagen I becomes a large component of the matrix [31], as does fibronectin, a protein which promotes dedifferentiation of vSMCs within the lesion [32]. Previous data from this laboratory indicate that collagen I restricts the ability of allylamine-activated cells to proliferate, while cells seeded on plastic or fibronectin continue to grow at faster rates than controls [22]. Collagen I may elicit a reduction in proliferative activity through changes in p21 and cyclin D1 expression [33], while fibronectin mediates increases in cellular proliferation. The underlying mechanisms that translate reciprocal interactions between integrins and extracellular matrix proteins to transcriptional responses remain to be fully elucidated. In healthy vessels, matrix interactions protect against hyperproliferation of vSMCs, while in injured vessels, as the composition of the matrix changes, integrin-coupled NF-κB activation and function is subject to significant modulation.

Matrix-dependent alterations in Rel protein expression were noted in allylamine vSMCs compared to controls, suggesting that NF-κB dimers in allylamine vSMCs at any given point differ from those in controls. Because each dimer may preferentially interact with a different subset of DNA domains [15], shifts in Rel protein expression may result in differential modulation of gene expression. This appears to be the case since differences in NF-κB DNA binding activity were observed for different promoters, and opn promoter activity was differentially regulated in allylamine cells. Shifts in dimer prevalence have been described during B-cell differentiation leading to altered transcriptional activation of the immunoglobulin κ light chain [34].

Progressive deletion analysis of the opn promoter uncovered strong repressor sequences within the promoter in vSMCs irrespective of phenotype. While the cis-acting elements responsible for gene regulation remain to be firmly established, several important findings were made. Notable differences between the two cellular phenotypes include (1) the activity of the opn promoter in allylamine cells was considerably lower than in control cells; (2) mutation of NF-κB, AP-1, USF-1, and VDRE elements significantly reduced luciferase activity in control cells, while in allylamine cells only mutation of VDRE exerted a negative effect; (3) mutation of NF-κB and USF binding sites in allylamine cells restored transcriptional activity to full-length control levels. Together, these findings suggest that opn promoter activity in allylamine cells is under negative control, possibly by NF-κB and USF-1 transcription factors. In fact, deletion of the NF-κB binding site slightly decreased promoter activity in control cells seeded on collagen I but did not affect the
positive regulatory influence of fibronectin on promoter activity, indicating that NF-κB may function as a positive or negative effector on opn promoter activity depending on phenotype.

Of note was the finding that opn promoter activity is influenced by the extracellular matrix on which the cells are seeded, with collagen exerting a repressor effect on promoter activity, and fibronectin affording a selective advantage to allylamine cells. This pattern of promoter activity parallels the proliferation and Rel protein expression profiles of control and allylamine cells, suggesting that the extracellular matrix dictates patterns of NF-κB and opn signaling in allylamine cells. Thus, signaling through integrin receptors bound to fibronectin participate in the regulation of opn promoter activity in allylamine cells. This is consistent with the upregulation of αvβ3 in allylamine cells seeded on a permissive matrix [23]. Regulation of opn expression in cells seeded on fibronectin does not seem to involve the NF-κB binding site located at −1943, while repression of promoter activity on collagen I may be partially accomplished through this NF-κB element. The ability of fibronectin to induce dedifferentiation of vSMCs has been well documented [32, 35], as well as its ability to induce expression of opn in cultured vSMCs.
opn is regarded as a critical modulator of atherogenesis, but little is known about the regulation of opn promoter in vSMCs. Interestingly, upstream stimulatory factor (USF1) has been implicated in injury-induced opn expression [36], while AP-1 has been implicated in regulation of opn [18], possibly as a result of increased ERK1/ERK2 activity [37]. Thus, NF-κB may serve as a regulator of the opn promoter in trans, an effect clearly dependent on the extracellular matrix. The repressor activity of NF-κB has been linked to the formation of p50/p52 heterodimers or p50/p50 homodimers [38, 39], a correlation that is consistent with the higher levels of these proteins observed in allylamine cells compared to controls, and the influence of fibronectin on patterns of protein expression. Since p50 and p52 contain no transactivation domain, hetero- and homodimers of these Rel proteins can bind NF-κB binding sites to inhibit transactivation [39, 40]. Indeed, a study of NF-κB binding sites suggests a high affinity of repressive NF-κB dimers for the opn promoter sequence. Interestingly, cooperation between USF-1 and NF-κB in the negative regulation of opn promoter activity is possible since mutation of either binding site restored promoter activity in allylamine cells to control levels, and cooperativity between these transcription factors in binding to nucleosomes has been described [41].

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