The Nuclear Factor κ-B Signaling Pathway Participates in Dysregulation of Vascular Smooth Muscle Cells in Vitro and in Human Atherosclerosis*

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In the lesions of atherosclerosis, vascular smooth muscle cells (SMC) display many functions characteristic of cytokine activation that likely contribute importantly to ongoing inflammation during human atherogenesis. The transcription factor nuclear factor κ-B (NFκB) often mediates the effects of cytokines on target cells, but the identity of Rel family members important in human SMC activation remains uncertain. In vitro, human SMC express multiple Rel family members. Of these, dimers of p65 and p50, but not a putative SMC-Rel, comprise basal and inducible NFκB binding activities. SMC express two inhibitor proteins IκBα and IκBo. Interleukin-1β stimulation caused transient loss of IκBo and a sustained decrease of IκBβ that correlated with increased and persistent levels of p65/p50 protein and binding activity in the nucleus. SMC cultured under serum-free conditions displayed little NFκB activity, but addition of serum or platelet-derived growth factor did activate NFκB. In situ analyses showed no evidence for basal NFκB activity in SMC in vivo as nonatherosclerotic arteries did not contain nuclear p65 or p50 protein. However, the nuclei of intimal SMC within human atheroma did contain both Rel proteins. We conclude that (i) dimers of p65 and p50, but not SMC-Rel, comprise NFκB complexes in human SMC; (ii) stimulatory components in serum activate NFκB and likely account for previously reported “constitutive” NFκB activity in cultured SMC; and (iii) exposure to inflammatory cytokines may produce prolonged NFκB activation in SMC because of sustained decreases in the inhibitory subunit IκBβ.

Vascular smooth muscle cells (SMC) at sites of atherosclerotic lesions express features of an inflammatory response, such as increased expression of genes encoding growth factor, inducible surface proteins, and molecules involved in extracellular matrix remodeling (1–3). The nuclear factor κ-B (NFκB) family of transcription factors has emerged as a regulator of many of these molecules by vascular cells. Inflammatory cytokines, oxidized lipids, and oxidative stress, factors or events present in human atheroma, can activate NFκB in vitro and also elicit specific functions in SMC (1, 4–6). Several genes up-regulated in SMC during atherogenesis, including vascular cell adhesion molecule 1 (VCAM-1), interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and c-myc also contain functional κB elements in their promoter/enhancer regions (5, 7). Moreover, SMC, macrophages, and endothelial cells within human atheroma exhibit nuclear localization of the NFκB subunit p65 (Rel A) in situ (8). Thus, in vitro and in situ studies underscore the potential importance of the NFκB pathway in dysfunction of vascular cells during atherogenesis.

NFκB exists in the cytosol of many cell types as an inactive complex of Rel-related factors, bound to a member of inhibitor proteins termed IκB (reviewed in Refs. 5 and 7). Rel family members include p65 (Rel A), Rel (c-Rel), Rel B, and the Dro sophila homolog dorsal, each of which contain transactivation domains necessary for gene induction. Other members, p50 (NFκB1) and p49 (NFκB2), are synthesized, respectively, as p105 and p100 precursors, and transactivate only weakly, but can form functional dimers with members of the first group. NFκB is sequestered in the cytosol in an inactive heteromeric complex by associating with one of several inhibitors denoted IκB, most commonly IκBα or IκBβ, or with Rel precursor proteins. Activation of NFκB follows phosphorylation of IκB or p105 on serine residues, possibly by a ubiquitin-regulated Ser/Thr kinase (9). Phosphorylated IκB or p105 is enzymatically degraded or processed to p50, respectively, by the multicatalytic proteasome complex (10), and liberated NFκB dimers then translocate to the nucleus and promote transactivation of target genes. One of these target genes encodes the inhibitor IκBα that binds to and thus limits further NFκB activity and gene expression (11, 12). IκBβ, another IκB member, is not resynthesized following its degradation, and may mediate prolonged NFκB activity in lymphocytes exposed to lipopolysaccharide or IL-1 (13). The diversity of dimeric complexes formed by Rel factors requires definition of the NFκB system in the context of each cell type examined.

In endothelial cells, inducible expression of leukocyte adhesion molecules requires participation of a well characterized NFκB system (14, 15). Bovine SMC were recently found to exhibit basal, constitutive NFκB activity in vitro (16). A novel, putative Rel protein termed “SMC-Rel” is thought to comprise constitutive NFκB activity in SMC and may permit cell division in serum-containing medium (16, 17). However, the repertoire of Rel proteins or their inhibitors expressed in SMC, the identity of NFκB members involved in DNA binding, or whether constitutive NFκB activity exists in human SMC in vivo has not been fully delineated. In view of the potential importance and unresolved issues regarding the role of NFκB in SMC, we
have addressed the identity of Rel family members and the issue of their constitutive expression in vitro and in vivo in human SMC cultures and in normal and diseased arterial specimens. As the inhibitory limb of control of NF-κB activity appears critical, we also tested the hypothesis that IκBα and IκBβ may play distinct roles in the control of cytokine activation of this cell type central to the pathogenesis of atherosclerosis.

MATERIALS AND METHODS

Antibodies—Rabbit affinity-purified polyclonal antiserum to NF-κB proteins p65, p50 (NFκB1), c-Rel, Rel B, IκBα/β-MAD-3, and IκBβ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody HIF-35 (mouse IgG1) recognizing muscle-specific actin was purchased from Enzo Diagnostics (Saysset, NY). Polyclonal rabbit neutralizing antibody to human PDGF-BB was purchased from Genzyme (Cambridge, MA). A nonimmune rabbit IgG fraction was from Dako (Carpenteria, CA).

Cell Culture—Smooth muscle cells obtained from explanted sections of human saphenous veins were grown in Dulbecco’s modified Eagle’s Medium (Life Technologies, Inc., Grand Island, NY) supplemented with 20 mM Hepes, 10% fetal calf serum (Hyclone), 5 mM L-glutamine, plus 50 units penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO2, 95% air. Cells were passaged by brief trypsinization and cultured to confluence (19) before addition to cell cultures.

Western Immunoblot Analysis—Whole cell lysates were prepared as described elsewhere (19) in 2 × SDS lysis buffer (1 × 125 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol). Equivalent amounts of whole cell lysates (30–40 μg of protein) or nuclear or cytosolic fractions (prepared as described below) were resolved on 10 or 12% SDS-PAGE gels, followed by electroblot transfer to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated in PBS-T (phosphate-buffered saline, 0.1% Tween-20), containing 5% non-fat dry milk for 1 h at 37 °C, and then incubated for 1 h with primary antibodies used at 0.4 μg/ml with the exception of IκBα antiserum, which was used at 0.5 μg/ml. Membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA), diluted 1:15,000 in PBS-T, 5% dry milk. Immunocomplexes were visualized using Renaissance chemiluminescence reagents (DuPont NEN) and exposure to x-ray film.

Preparation of Nuclear and Cytoplasmic Extracts and Electrophoretic Mobility Shift Assay—Saphenous vein SMC were grown to confluence (5–10 citt of cells) in 10-cm Petri dishes in serum-containing medium. In some experiments, cells were preincubated for 48 h in serum-free medium (Dulbecco’s modified Eagle’s/Ham’s F-12, 1:1, insulin 5 μg/ml, transferrin 5 μg/ml) to reduce exposure to serum components before addition to either 1 ml/well lysis buffer (100 mM KPO4, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol) and incubated for an additional 3 h at 37 °C. After three washes with ice-cold PBS, cells were maintained by two washes with ice-cold PBS and addition of 200 μl/well lysis buffer (100 mM KPO4, pH 7.8, 0.2% Triton X-100, 1 mM dithiothreitol). Luciferase and galactosidase activities were measured in 20-μl aliquots over a period of 5 s using the Tropix detection system (Bedford, MA). Relative luciferase activity was calculated by dividing luciferase by β-gal activity. For neutralization experiments, preparations of 10% FCS, 30 ng/ml PDGF-BB, or IT medium were incubated with or without 200 μg of PDGF-BB neutralizing antibody for 2 h at 37 °C prior to addition to cell cultures.

Indirect Immunofluorescence—Sections of human aortae and atherosclerotic carotid artery were obtained from transplantation donors and at endarterectomy, respectively. Paraaffin-embedded sections were deparaffinized with xylene and rehydrated with graded steps of ethanol, then incubated with 2.5% normal goat serum in PBS, p65c and p50 polyclonal antiserum or nonimmune rabbit IgG (1.5 μg/ml) diluted in 2.5% goat serum in PBS were added to sections for 2 h at room temperature. After three washes with PBS, sections were layered with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min. Complexes were detected with streptavidin-coupled Texas Red or fluorescein isothiocyanate (Amersham Corp.). Some sections were counterstained for cell nuclei with 0.5 mg/ml bis-benzimide (H-33258) (Calbiochem) in PBS. Antibody specificity for immunostaining was assessed by immunocytofluorescent staining of TNF-α-stimulated human saphenous vein endothelial cells with p65c and p50 antisera, with or without prior absorption with a 100-fold excess corresponding cognate peptides.

Fluorescence was observed using an Olympus BX60F microscope (Olympus Optical Co., Ltd., Japan).

RESULTS

Cultured Human SMC Express Predominantly NFκB p65 and p50 Species—Because of the unsettled nature of the identification of Rel subunits utilized by SMC we determined the spectrum of Rel family members expressed by saphenous vein SMC by immunoblot analysis of whole cell lysates. SMC contained prominent levels of p65 (Rel-A) compared with c-Rel or Rel B. c-Rel and Rel B in SMC comigrated with c-Rel and Rel B in Jurkat cell lysates and were abolished by preincubation with specific antiserum to p50κB (NFκB1) recognized a 50-kDa protein, suggesting that cultured SMC expressed p49 and p50 antisera, with or without prior absorption with a 100-fold excess corresponding cognate peptides.

Fluorescence was observed using an Olympus BX60F microscope (Olympus Optical Co., Ltd., Japan).

Biochemical Mechanisms of NFκB Signaling in Human Smooth Muscle Cells

In cultured SMC cultured in Serum-containing Medium exhib...
saphenous vein or aortic SMC following 2 h of incubation with or without IL-1β, a potent stimulus of NFκB activation. As the NFκB proteins involved in DNA binding have been identified in human endothelial cells (14), for comparison nuclear extracts were prepared from saphenous vein endothelial cells treated with or without TNF-α. Both venous and aortic SMC expressed two bands of specific DNA-binding complexes under unstimulated conditions, referred to as complexes I and II (Fig. 2a). Stimulation with IL-1β for 2 h markedly increased binding of complex I and slightly increased binding of complex II. Inclusion of polymyxin B (50 μg/ml) in the experiments did not inhibit binding activity, arguing against activation of NFκB by lipopolysaccharide contaminants (data not shown). In comparison to SMC, unstimulated endothelial cells showed little to no complex I and low levels of complex II, and binding activity was readily induced by stimulation with TNF-α. Of note, complexes I and II in SMC and EC comigrated in nondenaturing gels, suggesting a similar subunit composition. Unlabeled wild type NFκB probe inhibited binding activity to a much greater extent than mutant NFκB or GAS/ISRE probe, indicating the specificity of the DNA-binding complexes for NFκB motifs (Fig. 2b). A third, faster migrating complex (NS) likely represents a low affinity protein interaction with the NFκB probe used in these experiments as indicated by 1) lack of regulation by cytokine stimulation, 2) less competition or a smearing of the complex by cold competitor oligonucleotide, and 3) no interaction with recombinant IκBα added to the binding reaction (data not shown).

The subunit composition of basal and cytokine-induced DNA-binding complexes in human SMC was ascertained by supershifting with a panel of antisera to Rel family members (Figs. 3, a and b). Complex I in both unstimulated and IL-1β-stimulated cells interacted with two different antisera to the carboxyl or amino terminus of p65 (p65c and p65a, respectively), and with antisera to p50. Complex II interacted only with antisera to p50. Moreover, addition of both p65 and p50 antisera abolished all binding activity in nuclear extracts from unstimulated SMC. Antiseras to p49, c-Rel, Rel B, or nonimmune rabbit IgG did not affect either complexes I or II. Thus, in cultured human SMC, complex I likely contains heterodimers of p65 and p50, and complex II likely contains homodimers of p50. Human endothelial cells stimulated with TNF-α have identical subunit composition (14) (data not shown).

Loss of IκBα and Decreased IκBβ Correlates with NFκB Binding Activity and Nuclear Localization of Rel Proteins p65 and p50—NFκB signaling in cell types such as lymphocytes and macrophages is self-limited due to postinduction synthesis of the NFκB inhibitor IκBα (7); however, such an autoregulatory system has not been identified in SMC. To characterize further NFκB signaling in human SMC, the fate of inhibitor
proteins IκBα and IκBβ was followed over time in nuclear and cytoplasmic fractions from SMC stimulated with IL-1β or TNF-α. IκBα occurs as a ~37-kDa protein in the cytosol of unstimulated SMC (Fig. 4). IκBα rapidly (<0.5 h), yet transiently disappeared following stimulation with IL-1β or TNF-α, and returned to detectable levels at 2 h. The nuclear fraction contained no IκBα up to 24 h after cytokine stimulation, indicating that the disappearance of IκBα from the cytosol resulted from proteolysis rather than translocation to the nucleus. Inhibition of IL-1β- or TNF-α-induced depletion of IκBα by MG132, a potent inhibitor of proteasome activity, confirmed this interpretation (data not shown) (22). IκBβ occurs as a ~46-kDa protein in unstimulated SMC. Two hours following treatment with IL-1β, levels of IκBβ markedly decreased, but remained detectable. In contrast to IκBα, levels of IκBβ remained low throughout the period of treatment with IL-1β. By comparison, TNF-α affected levels of IκBβ little for up to 24 h (Fig. 4). Loss of cytosolic IκB correlated temporally with induction of NFκB binding activity in nuclear extracts from SMC cultured in parallel. Interestingly, the duration of increased NFκB activity correlated inversely with levels of IκBβ protein. Loss of cytoplasmic IκBβ in response to IL-1β associated with NFκB-DNA binding activity that persisted for 24 h. TNF-α stimulation, which affected IκBβ levels little, induced transient NFκB-DNA binding activity that peaked by 1 h and declined to near basal levels by 6 h (Fig. 4).

Consistent with the loss of IκB inhibitor proteins and increased NFκB binding activity, nuclear fractions contained increased amounts of p65 and p50 protein following IL-1β stimulation, determined by immunoblotting (Fig. 5). Increased levels of nuclear p65/p50 persisted for 24 h compared to unstimulated SMC, consistent with sustained NFκB binding activity observed during this time (Fig. 4). Nuclear extracts from unstimulated SMC contained low amounts of both p65 and p50, consistent with basal levels of DNA binding activity (Figs. 2 and 3). Levels of cytosolic p65 and p50 did not change appreciably during the experiments, suggesting that a small fraction of the total pool of these NFκB dimers translocated to the nucleus in response to cytokine stimulation. In striking contrast to the changes in p65 and p50, c-Rel remained cytosolic, and no nuclear accumulation of c-Rel was observed in response to IL-1β, indicating selectivity in mobilization of Rel dimers in human SMC.

**Serum Constituents Regulate NFκB Activity in Human Smooth Muscle Cells**—The above experiments examined NFκB family members in smooth muscle cells cultured in serum-containing medium. Since serum may contain or induce production of factors that can activate NFκB, experiments were performed in SMC cultured in a defined serum-free medium (IT medium). Removal of serum for 48 h strikingly decreased both basal NFκB-DNA binding as well as luciferase activity from a heterologous promoter construct containing three tandem repeat NFκB-binding elements (Fig. 6, a and b). Reintroduction of serum (10%) restored NFκB binding and luciferase activity to “basal” levels, which persisted for at least 24 h. Platelet-derived growth factor (PDGF), a serum-associated mitogen also expressed in human atheroma, alters many functions of SMC relevant in atherogenesis. PDGF-BB increased NFκB-DNA binding and luciferase activity in IT-cultured SMC, but did not further increase basal levels of NFκB-DNA binding in SMC maintained in serum (Figs. 6, a and b). A neutralizing PDGF...
antibody abolished the stimulatory effect of PDGF-BB on κB-dependent luciferase activity but failed to block the stimulatory effect of 10% serum (Fig. 6c), indicating that factors other than PDGF contribute to constitutive NFκB activity present in SMC cultured in serum. Human SMC responded similarly to IL-1β either in serum-free or serum-containing medium. Thus, “constitutive” NFκB activity in cultured human SMC likely results from the presence of serum constituents or, perhaps, the proliferative status of the cell population.

Expression of p65 and p50 in Normal and Atherosclerotic Human Vessels—In the normal vessel wall, SMC do not encounter mitogens or certain other constituents of serum, an unphysiologic fluid. We therefore tested whether SMC in the normal artery wall exhibit activation of NFκB. To this end, we examined sections of aortae obtained from transplantation donors for the presence of Rel proteins and, as described under “Materials and Methods.”

This study investigated the role of the NFκB signaling system in the regulation of functions of human SMC of importance in human atherosclerosis. Recent studies suggest that SMC in culture express basal, constitutive NFκB activity (16, 23). Prior to this, only lymphocyte cell lines and neurons were known to exhibit constitutive NFκB activity (24–26). Curiously, the Rel proteins that comprise this basal activity differ in each cell type. In lymphocytes and neurons, the basal complexes contain Rel-B heterodimers and p65/p50 dimers, respectively. In SMC, the basal complexes are thought to contain p50 and a putative Rel protein termed SMC-Rel (16). The results reported here suggest rather that the basal NFκB complexes in cultured human SMC contain p65/p50 heterodimers and p50/p50 homodimers, based on immunoblot and gel shift analyses. This discrepancy could reflect species differences between the bovine SMC used in the former study and the human SMC used herein. Indeed, bovine aortic SMC exhibit considerable levels of basal NFκB activity compared with human SMC, indicating that at least regulation of the NFκB system differs between these two cell types. Alternatively, SMC-Rel-containing complexes may not bind to the κ immunoglobulin enhancer motif used in the current experiments. This is unlikely, however, since basal NFκB activity in bovine SMC activated transcription from a reporter construct containing multimerized elements of the κ light chain enhancer (16). Moreover, experiments in human SMC using the tandem κB motif from the VCAM-1 promoter also identified basal complexes as containing p65 and p50 (23). Thus, the data suggest that, unlike bovine SMC, basal NFκB complexes in human SMC cultured in serum contain “classical” NFκB, i.e., p65 and p50.

Since the aforementioned studies examined SMC in culture, it is possible that culture conditions provoked a low level of basal NFκB activity, an activity lacking or not present in SMC of the vascular wall. Previous reports indicate that basal NFκB activity in cultured bovine SMC does not depend on serum growth factors (16). In human SMC, removal of serum considerably decreased “constitutive” NFκB-DNA binding as well as transcriptional activity from a reporter plasmid containing tandem κB-binding elements. NFκB activity was restored within hours upon reintroduction of either serum or PDGF, a growth factor considered important in atherogenesis. It is likely that serum constituents other that PDGF-BB chain contribute to the stimulatory effect of serum since a neutralizing PDGF-BB antibody failed to reduce serum-induced increases of NFκB activity, despite abolishing the stimulatory effect of PDGF. This finding suggests that constitutive NFκB activity in vitro results from exposure to serum, an unphysiologic medium, rather than being an intrinsic feature of SMC. Moreover, SMC exposed to growth factors such as PDGF may result in low level, persistent activation of NFκB that may contribute to sustained activation of this cell type at sites of vascular lesions (33). It should be noted that some residual NFκB activity remained even in SMC cultured without serum. This residual activity likely resulted from autocrine production of growth factors or IL-6, the latter of which is released from cultured SMC and can increase NFκB activity (17, 27). Nevertheless, these experiments cannot exclude this residual activity as basal NFκB activity in SMC in serum-free culture. A more definitive answer was provided by in situ analyses of human aorta from transplantation donors for the presence of Rel proteins p65 or p50. Nuclei within medial SMC showed no immunoreactivity with Rel antibodies, arguing against the presence of basal NFκB activity in SMC of the normal vessel wall. Together, the data support the view that NFκB is restricted to the cytosol in quiescent SMC, as occurs in most cell types examined, and that nuclear translocation of Rel proteins and increased NFκB activity occurs in response to cytokine as well as mitogenic stimulation.

2 T. Bourcier, G. Sukhova, and P. Libby, unpublished data.
In cell types such as macrophages and lymphocytes, activation of the NFκB system is self-limited due to increased synthesis of the NFκB inhibitor protein IκBa. Such an autoregulatory mechanism ensures transient activation of NFκB and circumvents a potential NFκB-mediated positive feedback loop of target gene expression in cells exposed to inflammatory stimuli. In SMC stimulated with IL-1β, increased levels of p65/p50 in the nucleus persisted for at least 24 h, despite the rapid reappearance of cytosolic IκBa to prestimulation levels. SMC thus appear to have a limited ability to curb NFκB activity induced by IL-1β. Another member of the IκB family may thus regulate NFκB in SMC. Indeed, the current results show that human SMC express the recently cloned NFκB inhibitor IκBb (13) and that treatment with IL-1β induces a sustained decrease in IκBb protein. Unlike IκBa, cytosolic pools of IκBb are not restored following activation and thus may result in persistent activation of NFκB. Indeed, sustained decreases in IκBb appear to cause persistent NFκB activity in T cells stimulated with IL-1 or lipopolysaccharide and in human endothelial cells treated with TNF-α (13, 28). Consistent with this view, TNF-α fails to modulate IκBβ levels substantially in human SMC during 24 h of incubation, whereas regulation of IκBa levels resembles that observed in response to IL-1β. In this case, increased NFκB activity is transient; indeed NFκB...
sustained NFκB activity may promote long term expression of gene products and maintain SMC in an activated or “primed” condition. In this regard, intimal SMC in atheroma show chronic expression of VCAM-1 (3, 33), an NFκB dependent process, and the current results show that NFκB is present in the nucleus of these cells.

Regulation of NFκB activity has emerged as a potentially important pathway in mediating specific functions of vascular endothelium pertinent to atherogenesis, such as expression of some adhesion molecules and PDGF (34). In vascular smooth muscle, activation of NFκB regulates expression of the adhesion molecule VCAM-1 and occurs during cell growth induced by serum or thrombin (17, 23, 35). The presence of the nuclear Rel protein p50 in intimal SMC within human atheroma shown here and of p65 shown here and elsewhere (8) support a role for this transcription factor in expression of SMC products of potential importance to progression of vascular lesions, including cytokines, growth factors, and proteins involved in coagulation. This study clarified the nature of the Rel proteins expressed in human SMC and characterized activation of this pathway in response to persistent proinflammatory stimuli, IL-1β and TNF-α. An understanding of the NFκB system in SMC should allow a more rational basis for elucidating potential roles of NFκB in mediating specific functions of SMC in vascular diseases.

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