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NF-κB is required for TNF-α-directed smooth muscle cell migration

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Abstract Migration of vascular smooth muscle cells (VSMC) is a crucial event in the formation of vascular stenotic lesions. Tumor necrosis factor-α (TNF-α) is elaborated by VSMC in atherosclerosis and following angioplasty. We investigated the role of nuclear factor-κB (NF-κB) in human VSMC migration induced by TNF-α. Adenoviral expression of a mutant form of the inhibitor of NF-κB, IκB-αM, suppressed TNF-α-triggered degradation of cellular IκB-α, inhibited activation of NF-κB, and attenuated TNF-α-induced migration. Further, IκB-αM suppressed TNF-α-stimulated release of interleukin-6 and -8 (IL-6 and IL-8). Neutralization of IL-6 and IL-8 with appropriate antibodies reduced TNF-α-induced VSMC migration. Addition of recombinant IL-6 and IL-8 stimulated migration. Collectively, our data provide initial evidence that TNF-α-mediated VSMC migration requires NF-κB activation and is associated with induction of IL-6 and IL-8 which act in an autocrine manner.

Key words: Tumor necrosis factor-α; Nuclear factor κB; Interleukin-6; Interleukin-8; Smooth muscle cell; Migration

1. Introduction

Migration and proliferation of vascular smooth muscle cells (VSMC) contribute to pathology of vascular stenotic diseases such as atherosclerosis and restenosis. Migration is accompanied by activation of multiple intracellular signaling pathways such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3′-kinase (PI3K) and focal adhesion kinase (FAK), modulation of the cytoskeleton, and alteration of the interaction of cell and extracellular matrix [1]. This complex response may be triggered by growth factors and chemotactic cytokines.

The nuclear factor-κB (NF-κB) plays a pivotal role in regulating expression of genes that influence cell differentiation, growth, and inflammation [2,3]. In most cell types under basal conditions, NF-κB is sequestered in the cytoplasm by one of a family of inhibitory proteins, such as IκB-α, and is inactive. NF-κB activation normally occurs after signal-initiated phosphorylation of IκB-α at serines 32 and 36 targets the inhibitor for rapid proteolysis [3]. Activated NF-κB translocates to the nucleus, binds to consensus sequences in promoter regions and initiates transactivation of responding genes [2,3]. The nuclear factor can be activated by a wide array of atherogenic stimuli such as growth factors and cytokines [3–5]. Activated NF-κB is present in atherosclerotic lesions but is undetected in normal arteries and is activated in VSMC after arterial balloon injury [5,6]. NF-κB activity was reported to be essential for cell proliferation and important in the formation of hyperplastic neointima [7–9]. Considerable attention has been focused on the regulation of VSMC mitogenesis by NF-κB but, to our knowledge, a role of NF-κB in VSMC migration has not been investigated.

Tumor necrosis factor-α (TNF-α), is expressed in atherosclerotic lesions and in the intima of arteries following injury or grafting but not in normal vessels [10,11]. In vivo experiments demonstrated a significant role of TNF-α in neointimal formation of vascular stenosis [12,13]. Migration and proliferation of VSMC are two crucial events in intimal hyperplasia. Reports on the ability of TNF-α to stimulate proliferation in cultured VSMC are conflicting and there are limited data defining the migration-stimulating activity of the cytokine [9,14–17]. TNF-α induces expression of numerous gene products including interleukins (IL-1β, IL-6 and IL-8), interferon-inducible protein 10, and extracellular matrix-degrading metalloproteinases (MMPs) [2,3,18,19]. Most of these products are involved in regulation of VSMC migration and proliferation [1,20–23]. TNF-α, like other chemoattractants such as platelet-derived growth factor and angiotensin [1], stimulates VSMC migration through the MAPK pathway [17]. The protein kinases may regulate multiple transcriptional activities of nuclear transcription factors such as activated protein-1 (AP-1), Ets and NF-κB [24–26]. However, unlike protein kinase pathways such as MAPK and PI3K, the role of transcription factors in VSMC migration is currently undetermined. NF-κB is a regulator of cytokines and potentially controls MMP-2 and MMP-9 [2,3,27,28]. Therefore, we investigated whether TNF-α-induced VSMC migration occurred though NF-κB activation.

In this study, we constructed a recombinant adenovirus vector expressing a mutated, stable form of IκB-α (IκB-αM). IκB-αM contains serine-to-alanine mutations at residues 32 and 36 which inhibit signal-induced phosphorylation and subsequent degradation of IκB-α [3,4]. The present work demonstrated for the first time that suppression of NF-κB activity led to a significant inhibition of TNF-α-directed human VSMC migration. Further, TNF-α-induced release of IL-6 and IL-8 and both modulated the migratory activity of TNF-α through an autocrine mechanism.

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2. Materials and methods

2.1. Cell culture

With IRB approval, VSMC were isolated from segments of internal mammary artery unused after coronary artery bypass, cultured, and identified by α-actin staining as previously described [29].

2.2. Cell migration assay

Migration was analyzed in transwell culture chambers with cells cultured on the upper surface of polycarbonate membranes coated with Matrigel® (Collaborative Research) as described [30]. Experiments were performed in culture medium plus 0.25% bovine serum albumin with recombinant human TNF-α, IL-6 (Genzyme) or IL-8 (Biosource) placed in the lower chamber for 12 h. Cells that migrated to the lower face of the membrane were quantitated with cresyl violet staining [31]. Cells without treatment served as control. In other experiments, anti-human IL-6 or IL-8 monoclonal antibody (Genzyme, Biosource) was also added to the upper and lower chambers. Normal mouse IgG (Sigma) was used an irrelevant control. Migration is expressed as fold increase over control.

2.3. Cell growth assay

Cell growth was assessed by methyltetrazolium (MTT) assay as described [32].

2.4. Construction of recombinant adenovirus vectors and infection of cells

Adenovirus vectors Adnull and Adβ-gal were constructed as previously described [33]. AdIκB-αM was constructed in a manner as described [34]. Adnull has no insert gene to be expressed and was used as control for effects of viral infection. Adβ-gal expressing the *Escherichia coli*. β-Galactosidase was used to estimate adenoviral gene transfer efficiency by X-gal staining of infected cells [35]. In preliminary experiments to establish optimal infection conditions, exposure of the VSMC to recombinant adenovirus at a multiplicity of infection of 200 for 40 min led to more than 90% gene transduction at 48 h. These conditions were used in the experiments with viral infection reported here.

2.5. Western blot analysis

IκB-α in whole cell lysates was detected by Western blot analysis.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed with a Promega gel shift system as previously described except for use of nuclear extracts instead of whole cell lysates [36]. Nuclear extracts were prepared as described [35].

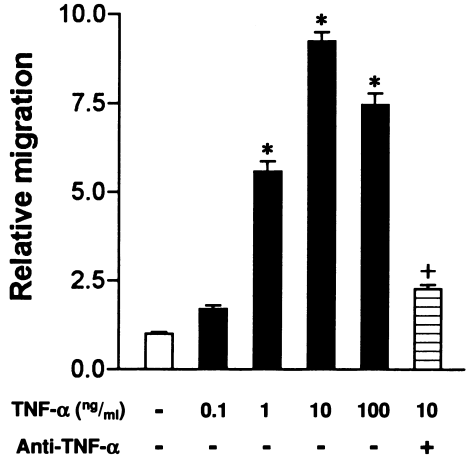

Fig. 1. Dose–effect of TNF-α on VSMC migration. After VSMC were exposed to TNF-α (0.1, 1, 10, or 100 ng/ml) for 12 h, migration was determined as described in Section 2. Migration is expressed as the fold increase relative to control. To determine the effect of an anti-TNF-α antibody on the ligand-induced VSMC migration, TNF-α (10 ng/ml) was preincubated with antibody for 30 min, and then the mixture was added to the lower chamber of the transwell. $^{*}P < 0.05$ vs. control. $^{+}P < 0.05$ vs. TNF-α (10 ng/ml) alone. Values are mean ± S.D. for three experiments

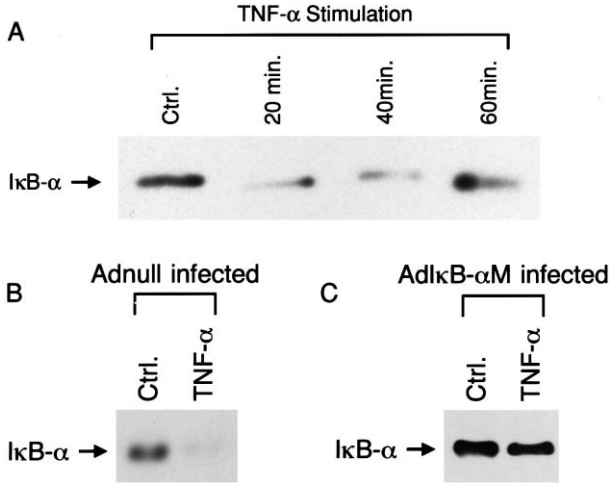

Fig. 2. Transient degradation of endogenous IκB-α by TNF-α and its prevention by expression of IκB-αM. A: Uninfected VSMC were treated with TNF-α (10 ng/ml) for the indicated periods of time. Whole cell lysates were prepared and analyzed by SDS–PAGE and immunoblotted with an anti-IκB-α antibody. B,C: Two days after infection with control virus, Adnull (B), or with AdIκB-αM (C), VSMC were treated with TNF-α (10 ng/ml). Whole cell lysates were analyzed by Western blot for cellular IκB-α. Shown is a representative of three individual experiments.

2.7. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure IL-6 and IL-8 in cell culture media.

2.8. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was performed by ANOVA with $P < 0.05$ considered to be significant.

3. Results and discussion

TNF-α-induced human VSMC migration in a concentration-dependent manner form 0.1–10 ng/ml with maximal activity at 10 ng/ml (Fig. 1). A 30 min preincubation with an anti-human TNF-α antibody (1.6 μg/ml) blocked the chemotactic activity of TNF-α (10 ng/ml). Therefore, 10 ng/ml TNF-α was used in all the following experiments unless otherwise specified. Similar to a previous report [9], our results from MTT assay or cell counting with trypan blue exclusion showed that treatment with TNF-α for 12 h at the concentrations used had no effect on cell proliferation (data not shown). Therefore, the possibility that proliferation affected migration was ruled out.

In order to evaluate the role of NF-κB in the regulation of TNF-α-mediated effects, we used a recombinant adenovirus vector expressing the NF-κB super-repressor, IκB-αM, to inhibit TNF-α-induced cellular IκB-α degradation and subsequent NF-κB activation. Initially, we determined the time-course of degradation of endogenous IκB-α induced by TNF-α. Treatment with TNF-α for 20 or 40 min led to degradation of IκB-α in uninfected cells (Fig. 2A). Resynthesis of IκB-α was detected by 60 min. Because of these results, all following measurements of IκB-α degradation and subsequent NF-κB activation were conducted at 40 min. We next determined the effects of IκB-αM expression on the cytokine-induced changes of IκB-α and NF-κB. TNF-α caused signifi-

cant reduction of IκB-α from constitutive levels in cells infected with Adnull (Fig. 2B). In contrast, IκB-α in cells infected with AdIκB-αM was largely resistant to the signal-stimulated degradation (Fig. 2C). In our experiments, EMSA analysis showed multiple sequence-specific bands representing NF-κB activity in both unstimulated cells and in cells stimulated by TNF-α. Basal activity of NF-κB in VSMC as well as multiple specific binding activity in EMSA seen here are consistent with several reports [7,37]. TNF-α augmented NF-κB DNA binding activity in cells infected with Adnull but not in VSMC infected with AdIκB-αM (Fig. 3).

Inhibition of NF-κB activation suppressed TNF-directed VSMC migration. TNF-α (10 ng/ml) increased migration greater than nine-fold in control cells and in cells infected with Adnull. In contrast, TNF-α-induced cell migration was only 2.7-fold in VSMC infected with AdIκB-αM (Fig. 4). The incomplete inhibition by expression of IκB-αM might imply that other signal pathways are involved in regulation of TNF-α-induced VSMC migration. Treatment with RNA synthesis inhibitor, actinomycin D (ActD, 1 μg/ml) or the protein synthesis inhibitor cycloheximide (CHX, 1 μg/ml) completely blocked TNF-α-induced cell migration. To ensure that the effects of viral infection or treatment with ActD or CHX on VSMC migration were not due to toxicity, VSMC were prepared and treated under the same experimental conditions as above. Cell viability was determined by trypan blue exclusion. There were no differences in cell viability among the experimental groups (data not shown). These results indicate that TNF-α chemotatic activity was dependent on a transcription mechanism in which transcription of NF-κB-responding genes played a dominant role.

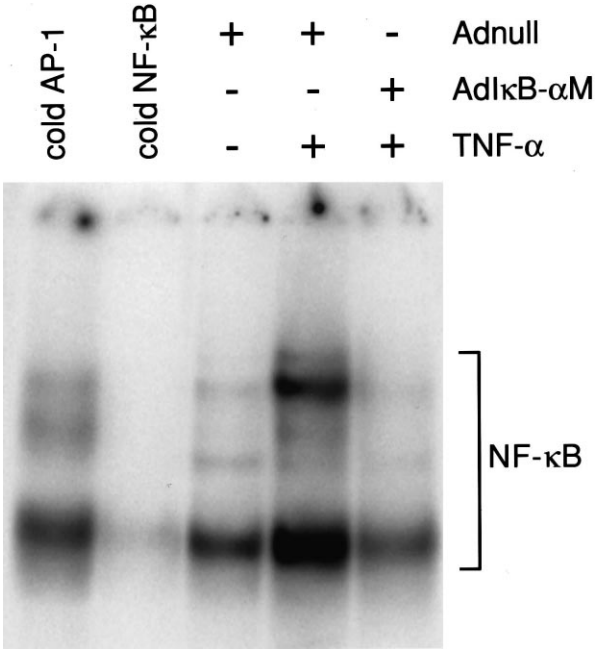

Fig. 3. Inhibition of TNF-α-elicited NF-κB activation by expression of IκB-αM. VSMC were infected with Adnull or AdIκB-αM and then stimulated with TNF (10 ng/ml) for 40 min. Nuclear extracts were prepared and analyzed by EMSA using NF-κB oligonucleotide. Molar excess of unlabeled NF-κB oligonucleotide or AP-1 oligonucleotide was used for competition as specificity control. A result of representative four individual experiments is shown.

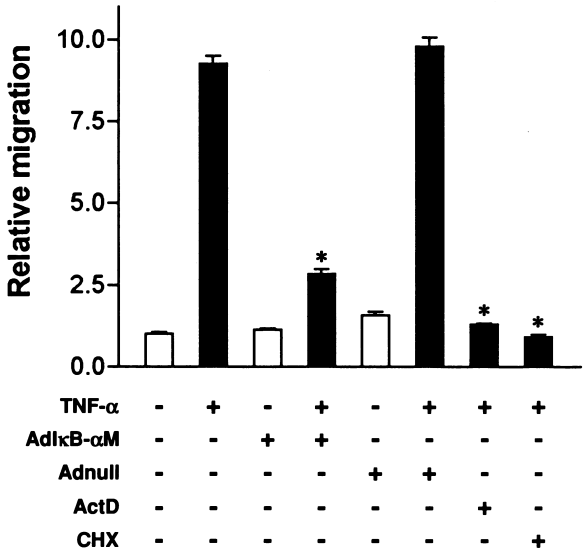

Fig. 4. Inhibition of NF-κB suppressed TNF-α-directed cell migration. Uninfected VSMC or VSMC infected with Adnull or AdIκB-αM were assayed for migration as in Fig. 1 after 12 h treatment with 10 ng/ml of TNF-α. In additional experiments, ActD (1 μg/ml) or CHX (1 μg/ml) was added 30 min prior to TNF-α. $^{*}P < 0.05$ vs. TNF-α alone or TNF-α+Adnull. Values are mean ± S.D. for three experiments.

In a variety of cell types, TNF-α may induce expression of NF-κB-responding gene products such as IL-6 and IL-8 that are mitogenic and/or chemotactic to VSMC [20,21]. We first detected the effect of TNF-α on IL-6 and IL-8 mRNA with Northern blot analysis. TNF-α increased transcription of genes encoding IL-6 and IL-8 within 1 h (data not shown). After treatment with TNF-α for 12 h, the time point that the

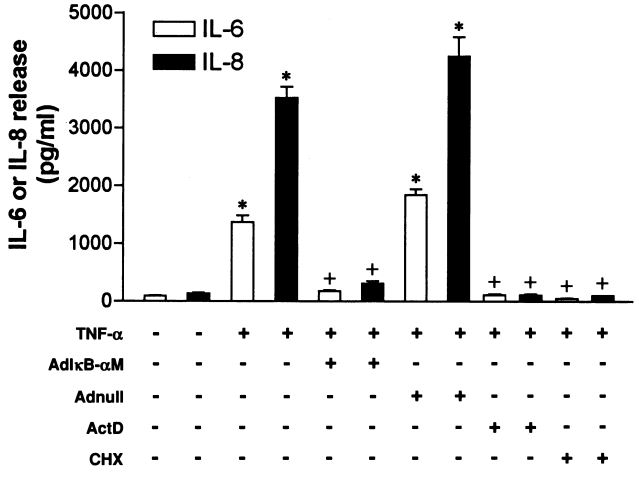

Fig. 5. TNF-α-induced release of IL-6 and IL-8 was inhibited by suppression of NF-κB. VSMC were prepared and treated as described in Fig. 4. Two days after infection with AdIκB-αM or Adnull, VSMC were stimulated with TNF-α (10 ng/ml) for 12 h. In additional experiments, ActD (1 μg/ml) or CHX (1 μg/ml) was added 30 min prior to TNF-α. Medium was collected and IL-6 and IL-8 concentration determined by ELISA. $^{*}P < 0.05$ vs. TNF-α alone or TNF-α+Adnull. $^{+}P < 0.05$ vs. TNF-α (10 ng/ml) alone or TNF-α+Adnull. Values are mean ± S.D. for six experiments.

migration experiment was terminated, the cells released IL-6 and IL-8 into the medium which was inhibited by infection with AdIκB-αM, but not the control virus vector, and by treatment with ActD (1 μg/ml) or CHX (1 μg/ml) (Fig. 5). To determine if IL-6 and IL-8 acted as autocrines in TNF-α-stimulated cell migration, we measured the effect of appropriate antibodies on the migration assay. As seen in Fig. 6, addition of anti-IL-6 (0.15 μg/ml) and/or anti-IL-8 (0.2 μg/ml) significantly attenuated TNF-α-induced migration. Simultaneous addition of both antibodies produced greater inhibition than either antibody alone. Addition of an irrelevant antibody at concentrations equal to the active was without effect (data not shown). To test whether IL-6 or IL-8 per se stimulated VSMC migration, we observed the effect of recombinant human IL-6 (1.5 ng/ml) or IL-8 (3.5 ng/ml), concentrations found in the medium of TNF-stimulated cells, on the cell migration. As shown in Fig. 7, both IL-6 and IL-8 significantly stimulated migration. IL-6 and IL-8 are likely not the only factors that are involved in TNF-α-directed, NF-κB-mediated migration of VSMC. This notion is supported by our observation that prevention of NF-κB activation inhibited cell migration by approximately 60% while addition of antibodies to IL-6 and IL-8 inhibited migration by about 30%. Such results indicate that other signaling factors are involved in TNF-α-directed migration. Collectively, These results indicated that released IL-6 and IL-8 functioned as secondary mediators contributing in part to the migratory activity of TNF-α. As previously stated, IL-8 is a chemoattractant for VSMC [21]. However, IL-6 regulation of VSMC migration has not been previously reported. Therefore, the data presented here provide new information about IL-6 activity in regulation of VSMC migration.

The mechanism by which NF-κB controls TNF-α's chemotactic activity may also involve activation of cytosolic protein kinases and degradation of the extracellular matrix by MMPs. TNF-α was found to induce expression or overexpression of MMP-1, -2, -3, and -9 in cultured VSMC [19]. Expression of MMP-2 and -9 appears to be regulated by NF-κB in endothelial cells and HT-1080 cells [27,28]. However, it remains to be determined if TNF-α regulates MMPs through NF-κB in VSMC. Additionally, multiple cytosolic signals such as cyclic AMP, MAPKs, FAK and PI3K are involved in the regulation of cell migration [1]. In this regard, it is reported that inhibition of MAPK activation attenuated TNF-α-mediated VSMC migration [17]. These multiple cytosolic signals might converge on NF-κB [9,26,38,39]. Therefore, NF-κB may play a pivotal role in regulating migration of VSMC induced by a number of stimuli but this remains to be investigated. Our results do not rule out the possibility of other transcription factors in regulation of the VSMC migration by TNF-α.

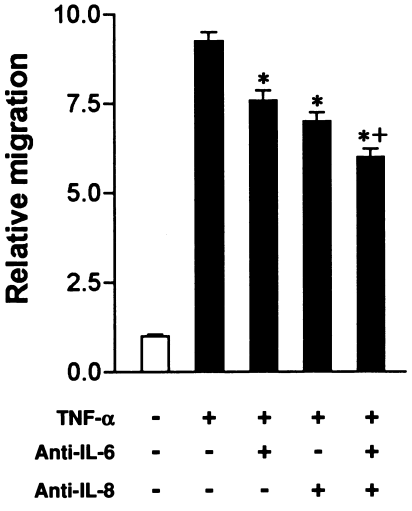

Fig. 6. Antibodies against IL-6 and IL-8 attenuated TNF-α-directed VSMC migration. VSMC were exposed to TNF-α (10 ng/ml) in the presence or the absence of an antibody against IL-6 or IL-8. After 12 h incubation, the migrated cells were quantitated. $*P < 0.05$ vs. TNF-α alone. Values are mean ± S.D. for three experiments.

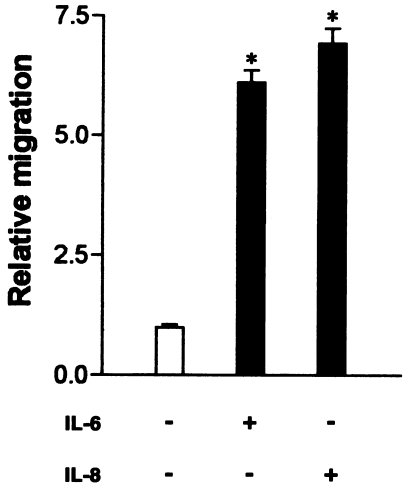

Fig. 7. Both IL-6 and IL-8 stimulated VSMC migration. The ability of IL-6 or IL-8 to stimulate VSMC migration was assessed after VSMC were exposed to IL-6 (1.5 ng/ml) or IL-8 (3.5 ng/ml) for 12 h. $*P < 0.05$ vs. control. Values are mean ± S.D. for three experiments.

In conclusion, our experiments showed for the first time that TNF-α, a cytokine expressed by VSMC in vascular disease, promoted VSMC migration through a mechanism dependent on the activation of NF-κB. TNF-α-induced expression of the NF-κB-responding cytokines, IL-6 and IL-8, and both partly contributed to the chemotatic activity of TNF-α. Additionally, the data presented here provide first evidence that IL-6 is a chemoattractant for VSMC. Collectively, these findings support the idea that activation of NF-κB plays a key role in regulating migration of VSMC.

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