Interleukin-11 attenuates human vascular smooth muscle cell proliferation

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Received 12 November 2001; accepted in final form 18 March 2002

Interleukin-11 (IL-11) is a multifunctional cytokine best known for its hematopoietic effects. Clinically, it is used to prevent chemotherapy-induced thrombocytopenia (5). IL-11 stimulates proliferation of human megakaryoblasts and osteoclasts in vitro (4, 8) and increases platelet counts and mucosal mass in vivo (2, 13). In addition to its growth factor influence, IL-11 also demonstrates anti-inflammatory properties. Low-dose IL-11 inhibits immune-mediated injury in cultured endothelium (12). Pretreatment of a mouse model of endotoxemia blocked lipopolysaccharide (LPS)-induced cytokine expression and protected against LPS-induced lung injury (22, 25). Trepicchio and colleagues (27) have suggested that these effects are mediated by blocking translocation of the transcription factor nuclear factor (NF)-κB.

Endovascular injury, as seen with angioplasty or a surgical anastomosis, promotes an inflammatory response that stimulates vascular smooth muscle cell (VSMC) proliferation and migration, ultimately resulting in intimal hyperplasia (17, 20). While VSMC are reportedly a source of IL-11 (24), the influence of IL-11 on VSMC physiology remains unknown. The purposes of this manuscript are to characterize the effect of IL-11 on 1) VSMC proliferation and 2) VSMC NF-κB activity.

MATERIALS AND METHODS

VSMC isolation and culture. Human VSMCs were isolated from segments of the thoracic aorta harvested from transplant donors as previously described (19). Phase contrast microscopy of cultured cells revealed typical “hill and valley” morphology. Purity of isolation was determined immunohis- tochemically with uniform phallodin staining for F-actin and α-smooth muscle actin (Sigma; St. Louis, MO) as well as lack of staining for the endothelial surface antigen von Willebrand factor. VSMC were nourished in a “complete medium” consisting of DMEM (Sigma), 5% fetal bovine serum (FBS; Summit Biotechnology; Ft. Collins, CO), human umbilical cord serum (graciously provided by Dr. Lawrence Horwitz, University of Colorado, Denver, CO), 0.01% MEM vitamins (Sigma), 10,000 U/ml penicillin G, 10,000 mg/ml streptomycin sulfate, and 25 mg/ml amphotericin (GIBCO-BRL; Grand Island, NY). “Serum-free medium” contained all of the above ingredients, with a serum component of only 0.5% FBS. Eight hours after the cells were plated, the media was changed to serum-free media for 48 h to allow for growth arrest in all experiments. VSMC were stimulated with recombinant basic fibroblastic growth factor (bFGF) with or without IL-11 (R&D Systems; Minneapolis, MN). These peptides were reconstituted in PBS with 0.5% BSA and diluted to desired concentration.

VSMC counting. VSMCs were seeded at a density of 2 × 10^4 cells/well on 24-well plates with complete media. Twenty
four hours after treatment, cells were washed twice with PBS and incubated with 200 μl of 0.05% trypsin for 5 min at 37°C. After trypsin was deactivated with 50 μl FBS, cells were aspirated into tubes and centrifuged at 500 g for 5 min. The supernatant was discarded, and cells were resuspended in 1 ml PBS. Cells were then directly counted using a hemocytometer.

**Mitochondrial assay.** Cells were plated with complete media in coated 96-well microtiter plates at a density of 3 × 10⁴ cells/well. Twenty-four hours after treatment, rates of proliferation were assayed by mitochondrial activity using the CellTitier 96 assay (Promega; Madison, WI). Methoxyphenyltetrazolium salt (MTS) compound is bioreduced by cells into a colored formazan product, which may be quantified colorimetrically. Cellular conversion occurs via NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. This technique is equivalent to tritiated thymidine incorporation in determining viable cell numbers (4). We have also demonstrated a linear correlation between MTS activity and cell numbers (19). Briefly, 20 μl of MTS/phena-zine ethosulfate were added to the appropriate wells, and plates were incubated at 37°C for 90 min. Absorbance was then recorded at 490 nm with a microtiter plate reader (Bio-Rad; Hercules, CA). Proliferation was subsequently expressed as absorbance (A).

**Immunohistochemistry.** VSMCs were plated in chambered tissue culture slides at a density of 2 × 10⁵ cells/well in complete medium. One hour after treatment, slides were washed once with warm PBS and fixed with 70% methanol-30% acetone for 10 min. After air drying, slides were washed three times in PBS for 10 min and blocked with 10% goat serum for 1 h at room temperature. Subsequently, cells were incubated at 4°C overnight with rabbit polyclonal anti-NF-κB p65 antibody (Santa Cruz Biotechnology; Santa Cruz, CA), 1:40 dilution with PBS-1% BSA. After three washes with PBS, cells were incubated in Cy3-labeled goat anti-rabbit IgG, 1:250 dilution, and Alexa-green wheat germ agglutinin-488 (Molecular Probes; Eugene, OR), 1:500 dilution, for 1 h in the dark at room temperature. After three washes, nuclei were stained with bis-benzimide (2.5 μg/ml). Fluorescent images were observed with the appropriate filter cubes and photographed using an automated confocal microscope under full software control by Intelligent Image Innovations.

**Nuclear extract preparation.** Nuclear extracts were prepared as previously described (1). Briefly, VSMC were plated at a density of 5 × 10⁶ cells/well and stimulated. Cells were harvested 1 h after stimulation and washed twice with ice-cold PBS. An aliquot of each sample was used for cell counting, and samples were centrifuged at 1,000 rpm for 10 min at 4°C. All samples were then incubated on ice for 15 min in buffer A [containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, and 10 mM KCl (pH 7.9)]. After the cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, the nuclei were centrifuged at 4°C for 6 min at 600 g. The nuclear pellet was then incubated on ice for 15 min in buffer C [containing 20 mM HEPES (pH 7.9), 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol], after which the extract was centrifuged at 4°C for 10 min at 12,000 g. The supernatants were collected, and the protein content was measured via a Coomassie protein assay (Pierce; Rockford, IL).

**NF-κB ELISA.** An ELISA was employed to investigate NF-κB activity in VSMCs (16). This assay is based on the specific binding of the active form of NF-κB from tissue extract to a NF-κB consensus site oligonucleotide attached to an ELISA plate. The primary antibody used to detect NF-κB recognizes an epitope of the p65 subunit, accessible only when NF-κB is activated and bound to its target DNA. A secondary horseradish peroxidase-conjugated antibody provides colorimetric readout quantified by spectrophotometry. Positive controls for the NF-κB p65 subunit were provided from cellular extracts previously evaluated by both ELISA and electrophoretic mobility shift assay (EMSA) (16) (Active Motif; Carlsbad, CA). To enhance the sensitivity of the ELISA assay, both wild-type and mutated consensus oligonucleotides were employed in each reaction.

VSMCs were plated at 5 × 10⁴ cells/well and treated as previously described. After the PBS wash and centrifugation, the supernatant was discarded, and samples were resuspended in 50 μl lysis buffer and incubated at 4°C for 10 min. Samples were then recentrifuged at 14,000 rpm for 20 min at 4°C. Supernatants were collected, and the protein content was measured via a Coomassie protein assay (Pierce). For whole cell lysates, 30 μg of total protein (per 5 × 10⁶ VSMCs) were loaded to each well and assayed by colorimetric absorbance according to the manufacturer’s directions (Active Motif). For intranuclear fractions, 25 μg of total protein (per 5 × 10⁶ VSMCs) were loaded to each well and assayed. Quantification of the NF-κB p65 subunit was expressed in mean absorbance (A) per sample.

**Cytokine assay.** Cells were plated at a density of 1 × 10⁵ cells/well and treated as above. After 24 h, VSMC supernatants were collected and the cells were counted. Samples were immediately assayed for IL-8 or IL-6 using liquid-phase electrochemiluminescence (ECL) (15). Briefly, purified polyclonal mouse anti-human IL-8 or IL-6 antibody (R&D Systems) was labeled with biotin (Ingen; Gaithersburg, MD). Antibodies were diluted to a final concentration of 1 mg/ml in PBS (pH 7.4) containing 0.25% BSA, 0.5% Tween 20, and 0.01% azide (ECL buffer). Biotinylated antibodies were incubated with a 1 mg/ml solution of streptavidin-coated paramagnetic beads (Dynal; Lake Success, NY) for 30 min at room temperature with vigorous shaking. Subsequently, it was combined with cell supernatants (25 μl) and goat monoclonal anti-human IL-8 (R&D Systems) previously labeled with ruthenium (Ingen). This mixture was shaken for an additional 2 h. The reaction was quenched with 200 ml ECL buffer, and the chemiluminescence was determined using an OriGene analyzer (Ingen). The detection limit was 40 pg/ml, and samples are reported in picograms per milliliter. IL-11 was measured in VSMC supernatants using a quan-tikine human IL-11 immunoassay ELISA kit (R&D Systems) after stimulation with bFGF (10 ng/ml), tumor necrosis factor (TNF)-α (10 ng/ml), platelet-derived growth factor (PDGF; 10 ng/ml), and LPS (10 ng/ml) (R&D Systems).

**Statistical analysis.** Data are presented as means ± SE. ANOVA (Stat View 5.0.1) with a Bonferroni-Dunn post hoc analysis was used to analyze differences between experimental groups. Statistical significance was accepted within 95% confidence limits.

**RESULTS**

**Effect of IL-11 on VSMC proliferation.** To determine the influence of IL-11 on VSMC proliferation, we performed direct cell counting in the presence of IL-11 for 24 h. At both 10 and 100 ng/ml, IL-11 had no direct effect on cell number compared with controls. This observation was not surprising in that we have previously demonstrated only moderate increases in growth between serum-free and complete media in our model (19). Therefore, we examined the effect of IL-11 after
stimulation with a prototypical atherogenic growth factor, bFGF. Incubation with 10 ng/ml bFGF resulted in increased VSMC growth compared with complete media controls (48,867 ± 1,700 vs. 18,024 ± 1,250 cells/ml, P < 0.05). Given concurrently, IL-11 inhibited bFGF-induced VSMC proliferation (Fig. 1). At a dose as low as 10 ng/ml, IL-11 decreased cell numbers compared with the mitogen-stimulated samples (15,205 ± 2,650 vs. 48,867 ± 1,700 cells/ml, P < 0.05). Similarly, IL-11 at 100 ng/ml decreased cell numbers compared with bFGF-stimulated cells (17,688 ± 1,959 vs. 48,867 ± 1,700 cells/ml, P < 0.05).

To verify that this observed decrease in cell number reflected viable and active VSMCs, we performed a cellular mitochondrial assay on bFGF-treated VSMCs with or without IL-11 (Fig. 2). bFGF-stimulated cells demonstrated a twofold increase in activity versus the complete media controls (1.75 ± 0.04 vs. 0.82 ± 0.07, P < 0.05). Compared with bFGF stimulation alone, IL-11 at 10 ng/ml inhibited proliferation (1.75 ± 0.04 vs. 1.05 ± 0.09, P < 0.05). IL-11 at 100 ng/ml had a similar effect on bFGF-induced proliferation (1.75 ± 0.04 vs. 1.17 ± 0.17, P < 0.05). IL-11 alone had no effect on VSMC proliferation compared with controls.

**IL-11 and NF-κB expression.** We utilized several methods to determine the influence of IL-11 on unbound NF-κB p65 protein. Qualitatively, immunofluorescence revealed an increased NF-κB p65 signal in the nuclei of bFGF-stimulated cells. IL-11 attenuated both cytosolic and nuclear signals in mitogen-stimulated cells yet had little effect on unstimulated VSMCs (Fig. 3). Quantitatively, an ELISA was employed to determine the amount of unbound NFκB p65 (λ) in each sample after stimulation (Fig. 4). In whole cell lysates, bFGF-stimulated samples demonstrated an eightfold increase in NFκB p65 compared with controls (2.79 ± 0.09 vs. 0.34 ± 0.12, P < 0.05). Compared with mitogen-induced samples, IL-11 (10 ng/ml) markedly decreased levels of NFκB p65 (0.94 ± 0.16 vs. 2.79 ± 0.34, P < 0.05). However, IL-11 did not return levels of NFκB p65 to control values. In intranuclear fractions, bFGF-stimulated VSMCs demonstrated a fourfold increase in p65 compared with controls (0.99 ± 0.08 vs. 0.25 ± 0.06). Similarly, IL-11 attenuated this response compared with bFGF-treated samples (0.58 ± 0.12 vs. 0.99 ± 0.08), although not to control levels. To monitor the specificity of the assay, both a wild-type and mutated p65-specific consensus oligonucleotide were used. When added to the reaction, the wild-type oligonucleotide consistently prevented p65 binding to the plate and resulted in zero absorbance at 450 nm. Conversely, the mutated consensus oligonucleotide had no effect.

**IL-11 and NF-κB-dependent cytokine expression.** To examine the downstream effect of IL-11 on VSMCs, we investigated the expression of two NFκB-dependent cytokines in cellular supernatants (Fig. 5). Quantitatively, bFGF-treated VSMCs produced eightfold more IL-8 compared with control cells (7.8 ± 0.9 vs. 67 ± 10 pg/ml, P < 0.05). IL-11 markedly decreased levels of IL-8 compared with bFGF-treated samples (30 ± 3 vs. 67 ± 10 pg/ml, P < 0.05). As seen with NFκB p65 levels, stimulated IL-8 levels did not completely return to the control level after IL-11 treatment. Similarly, IL-6 was increased 10-fold (297 ± 100 vs. 2,984 ± 225 pg/ml) by bFGF stimulation compared with controls. Again, IL-11 markedly reduced the IL-6 concentration compared with bFGF-treated samples (379 ± 185 vs. 2,894 ± 225 pg/ml).

**VSMC and endogenous IL-11 production.** We measured IL-11 in supernatants after mitogen and endotoxin stimulation. We were unable to detect any IL-11 in VSMC supernatants after stimulation with bFGF, TNF-α, or PDGF. LPS-treated cells did produce a modest amount of IL-11 (80 pg/ml). As such, endogenous IL-11 production likely did not influence our observations.
DISCUSSION

The majority of existing data suggest that IL-11 is a growth factor. In addition to previously mentioned reports (2, 4, 8, 13), mice treated with combination chemotherapy and radiation demonstrated a rapid recovery of small intestinal mucosa after IL-11 therapy (3). This recovery was associated with an increase in the mitotic index of crypt cells and an increase in proliferating cell nuclear antigen expression. Conversely, other reports suggest that IL-11 can act as an antiproliferative agent. Dose-dependent growth inhibition has been demonstrated in clinical tumor specimens (23). Furthermore, in rat intestinal cell lines, IL-11 increased cell doubling time, which was associated with a prolonged G1 phase and delayed entry into the S phase (14). To our knowledge, no study has examined the effect of IL-11 on VSMC proliferation.

In the present study, we demonstrated a decrease in human VSMC growth, as assayed by direct cell counting and mitochondrial activity, after treatment with IL-11 in vitro. It remains unclear if our differing results from previous reports are related to the experimental model or the specific cell lines studied. We did, however, observe consistent results using multiple human donors at various stages of passage. While one study reported IL-11 production from a cloned human VSMC cell line (24), we were unable to detect IL-11 in bFGF-treated human VSMC supernatants from donor specimens soon after harvest. This leads us to believe that endogenous IL-11 production is not responsible for these observations.

NF-κB is a transcription factor that mediates expression of numerous proinflammatory cytokines and growth factors. While it exists as a heterodimer in the cytoplasm, composed of both a p65 and a p50 subunit, p65 appears to be the dominant transcriptional activator. Vectors expressing either p65 alone or in combination with p50 effectively transactivates the c-myc promoter in vitro (9). Alternatively, p50 alone demonstrated only weak or no activity. In the present study, we measured free, unbound p65 levels. IL-11 has a profound negative effect on the expression of multiple inflammatory mediators after injury. IL-11-mediated suppression of NF-κB appears to be responsible for these anti-inflammatory effects both in vitro and in vivo (10, 27).

While we have demonstrated for the first time that IL-11 has a negative effect on VSMC proliferation and NF-κB activation, these data must be interpreted with several caveats. First, a wide array of inflammatory mediators are implicated in neointimal hyperplasia and atherogenesis. bFGF as a representative of the growth factor family is released from a variety of inflammatory cells and has well-established proliferative effects in vitro and in vivo (11, 18). While we have focused our studies on bFGF, we acknowledge that no single cytokine or growth factor is likely responsible for vascular remodeling.

Second, we recognize the use of EMSA as a well-known standard for measuring NF-κB. However, recent data suggest that the ELISA assay is more sensitive compared with EMSA in vitro (16). Furthermore, whereas most EMSA is performed on nuclear fractions, we were able to measure both total cellular and intranuclear unbound NF-κB p65 protein. As measured, cytosolic NF-κB p65 is still important as it is free from...
its heterodimer but presumably not yet translocated. We detected elevated levels of p65 in both the cytosol and nuclei of bFGF-treated cells. Interestingly, IL-11 appeared to decrease cytosolic levels to a relatively higher degree than that observed with the intranuclear levels.

We can only speculate as to the proposed antiproliferative mechanism of IL-11. We have previously demonstrated a strong relationship between NF-κB activity and VSMC proliferation (21). NF-κB may act as a direct proliferative influence or may promote transcription of other mitogens. We examined IL-8 and IL-6 as prototypical downstream NF-κB products. While best known as a chemoattractant, IL-8 can also stimulate VSMC growth (28). Similarly, IL-6 has been shown to promote VSMC proliferation in an autocrine fashion (6, 21). However, the influence of IL-11 on NF-κB is not likely the full story. In fact, we demonstrated that IL-11 is unable to completely reverse bFGF-induced NF-κB activation and IL-8 secretion. This observation is not surprising in that bFGF relies on tyrosine kinase receptor signaling, which can promote VSMC proliferation independently of its effects on NF-κB (7). Furthermore, as a member of the gp130 cytokine receptor family (26), IL-11 likely mediates several NF-κB-independent intracellular events.
REFERENCES


