Nitric Oxide Mediates Cytotoxicity and Basic Fibroblast Growth Factor Release in Cultured Vascular Smooth Muscle Cells

A Possible Mechanism of Neovascularization in Atherosclerotic Plaques

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Abstract

To define the pathophysiological role of nitric oxide (NO) released from vascular smooth muscle cells (VSMC), we examined whether NO released from VSMC induces cytotoxicity in VSMC themselves and adjacent endothelial cells (EC) using a coculture system. Prolonged incubation with interleukin-1 (IL-1) induced large amounts of NO release and cytotoxicity in VSMC. N^G-Monomethyl-L-arginine, an inhibitor of NO synthesis, inhibited both NO release and cytotoxicity induced by IL-1. In contrast, DNA synthesis in cocultured EC was not inhibited but rather stimulated by prolonged incubation with IL-1 or sodium nitroprusside (SNP), a NO donor. However, IL-1 and SNP did not stimulate but inhibited DNA synthesis in EC alone. On the other hand, conditioned medium from VSMC incubated for a long period with IL-1 or SNP stimulated DNA synthesis in EC alone. Furthermore, the concentration of basic fibroblast growth factor in the conditioned medium was increased and correlated with the degree of cytotoxicity in VSMC. These results indicate that NO released from VSMC induces VSMC death, which results in release of basic fibroblast growth factor, which then stimulates adjacent EC proliferation. Thus, NO released from VSMC may participate in the mechanism of neovascularization in atherosclerotic plaques. (*J. Clin. Invest.* 1995. 95:669–676.) Key words: interleukin-1 • atherosclerosis • endothelial cell • cellular interaction

Introduction

The early lesion of atherosclerosis is an aggregation of lipid-rich macrophages within the intima (1). The characteristic lesion of advanced atherosclerosis is the fibrous plaque which consists of a necrotic core of cellular debris, lipid, cholesterol, and calcium deposits surrounded by vascular smooth muscle cells (VSMC),[1] macrophages, and T lymphocytes (2). Neovascularization is often seen within fibrous plaques (3).

Since macrophages are present in almost all stages of atherosclerotic lesions (4), cell–cell interactions among macrophages, endothelial cells (EC), and VSMC may be critical in modifying the progression of atherosclerosis. Through a network of cellular interactions, cytokines and growth factors released from these cells may stimulate their neighbors in a paracrine manner, or themselves in an autocrine manner (5).

Basic fibroblast growth factor (bFGF), which is a strong mitogen for both EC and VSMC, is present in the cytosol of these cells (6). Although bFGF has no signal sequence, it may be released as a result of cell injury. Balloon catheter injury of an artery is typically followed by proliferation of VSMC with subsequent formation of an intimal thickening (7). There are reports that suggest that endogenous bFGF is responsible for the initiation of VSMC proliferation seen after vascular injury (8–10). Since cell necrosis and increased cell turnover are found in atherosclerotic lesions (11, 12), release of stored bFGF from injured vascular cells may be an important mediator in the progression of atherosclerotic lesions.

Nitric oxide (NO) is an unstable but multifunctional molecule which mediates a number of diverse physiological processes, such as smooth muscle relaxation, macrophage cytotoxicity, and neurotransmission (13, 14). At least three distinct forms of NO synthase (NOS) have been cloned and characterized (15). The macrophage enzyme has been referred to as 'inducible NOS' (iNOS), whereas the enzymes in neuronal tissue and endothelium appear to be expressed constitutively. The constitutive NOS system generates only small amounts of NO for short periods of time. In contrast, the iNOS system begins to generate large amounts of NO several hours after exposure to cytokines. There is recent evidence that interleukin-1 (IL-1), a macrophage-derived cytokine, stimulates the release of large amounts of NO from VSMC in vitro (16, 17). Joly et al. (18) also suggested that vascular smooth muscle contains a system that generates NO after balloon injury in vivo. Furthermore, Nunokawa et al. (19) have recently cloned iNOS in VSMC. However, the pathophysiological roles of this NO released from VSMC have not been defined.

Since NO is known to exert various toxic effects on many cell types (20), we examined the possibility that large amounts of NO released from VSMC may mediate cytotoxicity in VSMC

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; EC, endothelial cells; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; LDH, lactate dehydrogenase; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cells.

and adjacent EC. We found that IL-1 induced a time-dependent release of high levels of NO from VSMC and cytotoxicity in VSMC themselves but not in adjacent EC. Furthermore, damaged VSMC released stored bFGF which stimulated adjacent EC proliferation.

Methods

Materials. Human recombinant IL-1β was donated by Otsuka Pharmaceutical Co. (Tokushima, Japan). N^G-Monomethyl-L-arginine (L-NMMA), sodium nitroprusside (SNP), and L-arginine (L-Arg) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine (5 Ci/mmol) was from Amersham International (Bucks, United Kingdom). Mouse monoclonal antibody against human bFGF was prepared as described previously (21, 22).

Cell culture. VSMC were isolated from Wistar rat aortas by explant method as described previously (23) and grown in Dulbecco's modified Eagle's medium (DME) containing 10% FCS. The cells exhibited typical 'hill and valley' growth morphology and almost all cells reacted with the anti-α-actin monoclonal antibody (Boehringer Mannheim, Mannheim, Germany). For experiments, VSMC (passages 4–9) were grown to confluence on 12- or 24-well plates with DME supplemented with 10% FCS.

Bovine aortic EC were isolated following the method of Jaffe (24). EC were harvested after light collagenase digestion of the intimal layer of the thoracic aorta. Cells were then seeded initially into 60-mm collagen-coated dishes with DME supplemented with 10% FCS and were identified as EC by typical cobblestone appearance and Factor VIII immunofluorescence.

For coculture system, EC (passages 4–8) were grown to confluence on the collagen-coated microporous membrane of transwells (12-mm diameter, 3.0-μm pore size; Costar Corp., Cambridge, MA). After reaching confluence, VSMC were preincubated with serum-free DME containing 0.1% bovine serum albumin (BSA) to become quiescent. Then the coculture system was prepared by placing the transwells into the wells of the culture plate, and both cells were cultured in DME containing 0.1% BSA in the presence or absence of 1 nM IL-1 or other compounds. In this coculture system, the culture medium was shared by both cells. Therefore, humoral interchange was allowed between them without direct cell contact.

Nitrite assay. Since NO in aqueous solution containing oxygen is oxidized primarily to NO_2^- (nitrite) with little or no formation of NO_3^- (nitrate) (25), we measured the level of nitrite in the medium as a reflection of NO production. The nitrite level in cell-free supernatant was determined using Griess reagent consisting of 1% sulfanilamide, 0.1% naphthylethylene-diamine-dihydrochloride, and 2% H_3PO_4. Briefly, 100 μl culture supernatant was mixed with 100 μl Griess reagent and incubated in a 96-well plate for 15 min at room temperature. Nitrite concentration, proportional to OD_{540}, was determined using a microtiter plate reader (model 450; Bio-Rad Laboratories, Inc., Tokyo, Japan) with reference to $NaNO_2$ as a standard.

Determination of DNA synthesis in VSMC. Quiescent VSMC cultured in 24-well plates or VSMC cocultured in 12-well plates with EC were incubated for the indicated times with 1 nM IL-1 in the presence or absence of 3 mM L-NMMA. Cells were then labeled with [^{3}H]-thymidine (2 μCi/ml) during the last 4-h period of the incubation. After the labeling, cells were then washed three times with cold phosphate-buffered saline (PBS) and treated with 5% trichloroacetic acid and ethanol-ethylether (3:1, vol/vol). The residues in the wells were solubilized in 0.3 N NaOH, and the radioactivity of aliquots of the solution was measured after the neutralization of pH.

Determination of lactate dehydrogenase (LDH) activity. Confluent VSMC cultured in 24-multiwell plates were preincubated with serum-free DME containing 0.1% BSA for 48 h. Cells were then incubated for the indicated times with various compounds. After the incubation, the medium was collected and stored at −20°C until assay. LDH activity in 200-μl aliquots of cell-free supernatant was measured spectrophotometrically at 340 nm using a commercial kit (Sigma Chemical Co.). Total LDH activity was determined from the supernatant of a sample treated with 0.1% Triton X-100 for 30 min.

Determinations of DNA synthesis in EC. Confluent EC cultured on the collagen-coated microporous membrane of transwells were cocultured for 72 h with VSMC in serum-free medium containing SNP or IL-1 in the presence or absence of L-NMMA. Confluent EC cultured in 24- or 96-well plates were incubated for 24 h with serum-free DME containing various compounds or conditioned medium from VSMC. Cells were labeled with [^{3}H]thymidine (2 μCi/ml) during the last 4-h period of the incubation. After the labeling, EC cultured on the plates were then treated by the same method as for determination of DNA synthesis in VSMC. EC, which were cultured on the collagen-coated membrane of the transwells, were washed three times with cold PBS, then cells were solubilized in 0.3 N NaOH, and the radioactivity of aliquots of the solution was measured after the neutralization of pH.

Immunocytochemistry. Sparse VSMC grown in 2-chamber culture dishes (Lab-Tek, Nunc, Inc., Naperville, IL) were fixed for 5 min with 5% acrolein in PBS at room temperature. The cells were washed twice for 5 min each with PBS, then incubated with 1% normal goat serum/PBS for 20 min at room temperature. After washing with PBS for 5 min, cells were incubated for 24 h at 4°C with primary antibody against bFGF (mouse anti-human bFGF monoclonal antibody) diluted in PBS containing 0.1% Triton X-100 and 1% normal goat serum at a concentration of 10 μg/ml. After the incubation, cells were washed four times for 20 min each with PBS containing 0.1% Triton X-100 at room temperature. Then cells were incubated with biotinylated goat anti-mouse IgG overnight at 4°C. The avidin-biotin-peroxidase complex (ABC) solution was prepared according to the protocol of Vectastain ABC kit (Vector Labs, Inc., Burlingame, CA). The ABC solution was then added, and incubation proceeded overnight at 4°C. After another wash with 0.05 M Tris/HCl buffer (pH 7.4) for 30 min, the cells were reacted with 0.02% diaminobenzidine and 0.005% hydrogen peroxide substrate in Tris/HCl buffer for 5 min at room temperature. The cells were then washed three times for 5 min each with Tris/HCl buffer, dehydrated, and coverslipped with Permount (Merk Japan Ltd., Tokyo, Japan).

Determination of bFGF. Conditioned medium from VSMC incubated with vehicle, 0.1 mM SNP, or 1 nM IL-1 in the presence or absence of 3 mM L-NMMA was obtained as described in the method of LDH determination. The concentration of bFGF in the conditioned medium was determined using an EIA kit (Amersham International).

Statistical analysis. Statistical analysis was performed by one-way analysis of variance. Results are expressed as mean±SEM. A value of $P < 0.05$ was considered significant.

Results

Prolonged incubation with IL-1 induces high levels of NO release and inhibition of DNA synthesis in VSMC. Fig. 1 shows the kinetics of NO production and [^{3}H]thymidine incorporation in VSMC after exposure to 1 nM IL-1. NO release from VSMC was determined by measuring nitrite level in the culture supernatant. IL-1 induced a time-dependent and high level of NO release from VSMC. Coincubation with 3 mM L-NMMA, an inhibitor of NO synthesis, at a higher concentration than that of L-Arg in the medium (0.4 mM), significantly inhibited IL-1–induced NO release from VSMC (Fig. 1 *A*). On the other hand, IL-1 induced a time-dependent but biphasic effect on [^{3}H]thymidine incorporation in VSMC (Fig. 1 *B*). Short incubation for 24 h with IL-1 induced stimulation of [^{3}H]thymidine incorporation in VSMC, whereas prolonged incubation with IL-1 for 58 and 72 h induced significant inhibition of [^{3}H]-thymidine incorporation in VSMC. However, coincubation with L-NMMA prevented the inhibition induced by prolonged incubation with IL-1. Furthermore, in the presence of L-NMMA, IL-1 induced only stimulation of [^{3}H]thymidine incorporation

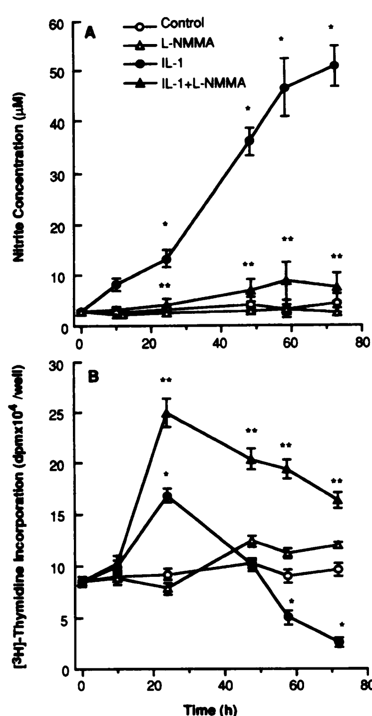

Figure 1. Kinetics of NO release and [^{3}H]-thymidine incorporation after stimulation of VSMC with IL-1. Confluent VSMC were preincubated for 48 h with DME containing 0.1% BSA to make cells quiescent, then cells were incubated in new medium containing IL-1 (1 nM) in the presence or absence of L-NMMA (3 mM) for the times indicated. After collection of the incubation medium, VSMC were pulse-labeled during the last 4 h of the incubation period. Nitrite concentration in the medium (*A*) and incorporation of [^{3}H]-thymidine (*B*) were determined as described in the text. Values are mean±SEM of four individual experiments containing three replicates in each experiment. *$P < 0.05$, significantly different from control. **$P < 0.05$, significantly different from cells treated with IL-1 alone.

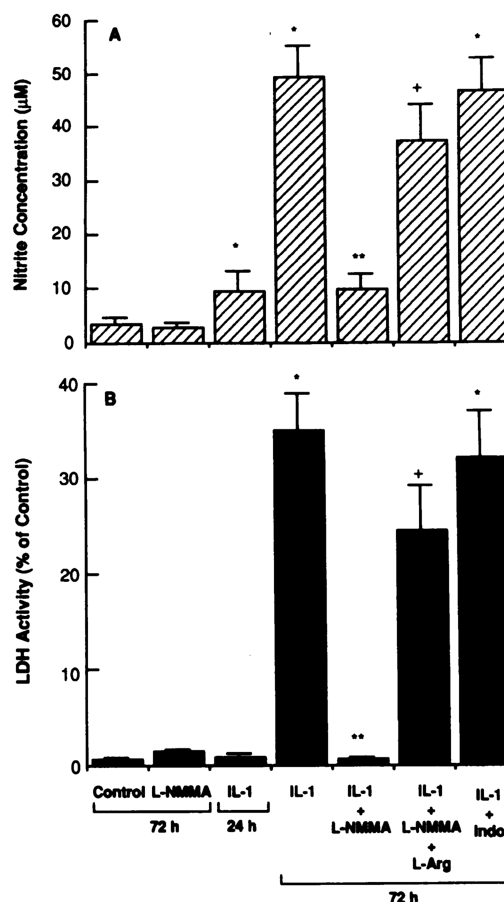

Figure 2. Effects of IL-1 on NO and LDH release from VSMC. Quiescent VSMC were incubated for 24 or 72 h with serum-free medium containing IL-1 in the presence or absence of L-NMMA, L-Arg, or indomethacin (*Indo*). After the incubation, medium was collected and stored at −20°C until assay. Nitrite concentration (*A*) and LDH activity (*B*) in the medium were determined as described in the text. Values are mean±SEM of four different experiments containing two replicates in each experiment. *$P < 0.05$, significantly different from control. **$P < 0.05$, significantly different from cells treated with IL-1 alone. $^+P < 0.05$, significantly different from cells treated with IL-1 and L-NMMA.

in a time-dependent manner. Maximal response was detected at 24 h with IL-1 in the presence of L-NMMA (Fig. 1 *B*).

Prolonged incubation with IL-1 stimulates LDH release from VSMC. Prolonged incubation for 72 h with IL-1 induced significant stimulation of LDH release from VSMC (Fig. 2 *B*), whereas short incubation for 24 h with IL-1 did not induce LDH release. However, L-NMMA significantly inhibited the IL-1–induced stimulation of both NO and LDH release from VSMC. The inhibition of both NO and LDH release induced by 3 mM L-NMMA was partially but significantly prevented by coincubation with 30 mM L-Arg. However, 10^{-6} M indomethacin, a cyclooxygenase inhibitor, did not affect IL-1–induced NO or LDH release from VSMC (Fig. 2, *A* and *B*). The degree of LDH release was correlated with the degree of NO release from VSMC after stimulation with IL-1.

Prolonged incubation with IL-1 induces stimulation of DNA synthesis in EC when EC are cocultured with VSMC. We next examined the effects of prolonged incubation with IL-1 on VSMC and adjacent EC when VSMC were cocultured with EC without direct cell contact. As shown in Fig. 3, *A* and *B*, prolonged incubation for 72 h with IL-1 induced high levels of NO release from VSMC cocultured with EC to the same degree as in VSMC alone. It also induced significant inhibition of DNA synthesis in cocultured VSMC. L-NMMA prevented NO release and inhibition of DNA synthesis in cocultured VSMC induced by prolonged incubation with IL-1. SNP, a NO donor, also induced high levels of NO release and inhibition of DNA synthesis in cocultured VSMC.

On the other hand, prolonged incubation with IL-1 did not inhibit but rather stimulated DNA synthesis in EC cocultured with VSMC (Fig. 4). Coincubation with L-NMMA inhibited IL-1–induced stimulation of DNA synthesis in cocultured EC. Furthermore, incubation with SNP also stimulated DNA synthesis in cocultured EC (Fig. 4).

IL-1 and SNP inhibit DNA synthesis in EC alone. Incubation for 24 h with IL-1 did not affect NO release from EC but it significantly inhibited DNA synthesis in EC. L-NMMA did not affect the IL-1–induced inhibition of DNA synthesis in EC. Incubation with 0.1 mM SNP also inhibited DNA synthesis in EC (Fig. 5, *A* and *B*). Furthermore, prolonged incubation of EC for 72 h with serum-free medium induced cell detachment and cell death (> 90% of EC were stained with 0.1% trypan blue, data not shown).

Conditioned medium from VSMC incubated with IL-1 stimulates DNA synthesis in EC. We next examined the effect of conditioned medium from IL-1–treated VSMC on DNA synthesis in EC (Fig. 6). Conditioned medium from VSMC incubated with or without IL-1 in the presence or absence of L-NMMA for various times up to 96 h was added to EC and incubated

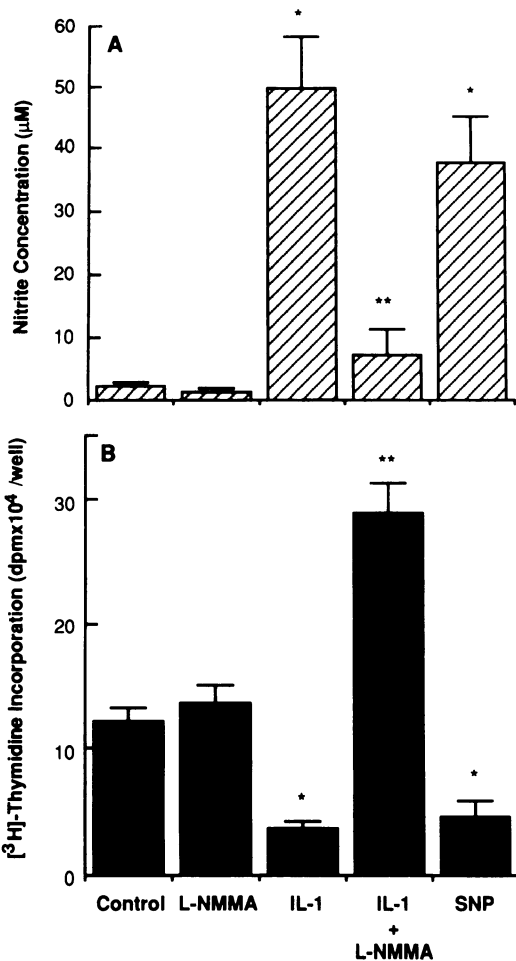

Figure 3. Effects of IL-1 on NO release and DNA synthesis in VSMC cocultured with EC. Quiescent VSMC were cocultured with EC as described in the text. Cocultured cells were then incubated for 72 h with serum-free medium containing SNP or IL-1 in the presence or absence of L-NMMA. Nitrite concentration in the medium (*A*) and [^{3}H]thymidine incorporation (*B*) were determined as described in the legend to Fig. 1. Values are mean±SEM of four different experiments containing two replicates in each experiment. $*P < 0.05$, significantly different from control. $**P < 0.05$, significantly different from cells treated with IL-1 alone.

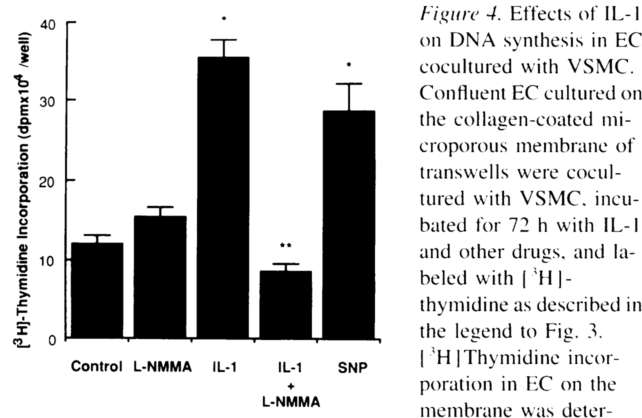

Figure 4. Effects of IL-1 on DNA synthesis in EC cocultured with VSMC. Confluent EC cultured on the collagen-coated microporous membrane of transwells were cocultured with VSMC, incubated for 72 h with IL-1 and other drugs, and labeled with [^{3}H]-thymidine as described in the legend to Fig. 3. [^{3}H]Thymidine incorporation in EC on the membrane was determined as described in the text. Values are mean±SEM of four different experiments containing two replicates in each experiment. $*P < 0.05$, significantly different from control. $**P < 0.05$, significantly different from cells treated with IL-1 alone.

for 24 h. [^{3}H]Thymidine was labeled during the last 4-h period of the incubation. DNA synthesis in EC incubated with the conditioned medium from IL-1–treated VSMC was significantly more enhanced than that from control VSMC (Fig. 6). The degree of stimulation of DNA synthesis by the conditioned medium was dependent on the duration of incubation with IL-1. However, conditioned medium from VSMC incubated with IL-1 in the presence of L-NMMA did not enhance DNA synthesis (Fig. 6).

Increase in DNA synthesis induced by conditioned medium from IL-1–treated VSMC was partially inhibited by treatment with bFGF neutralizing antibody. To examine whether bFGF, an EC mitogen, is involved in the mitogenic action of conditioned medium from IL-1–treated VSMC, we used monoclonal antibody against bFGF to specifically neutralize its mitogenic effect. Coincubation with 10 μg/ml anti-bFGF IgG did partially neutralize the stimulation of DNA synthesis in EC induced by

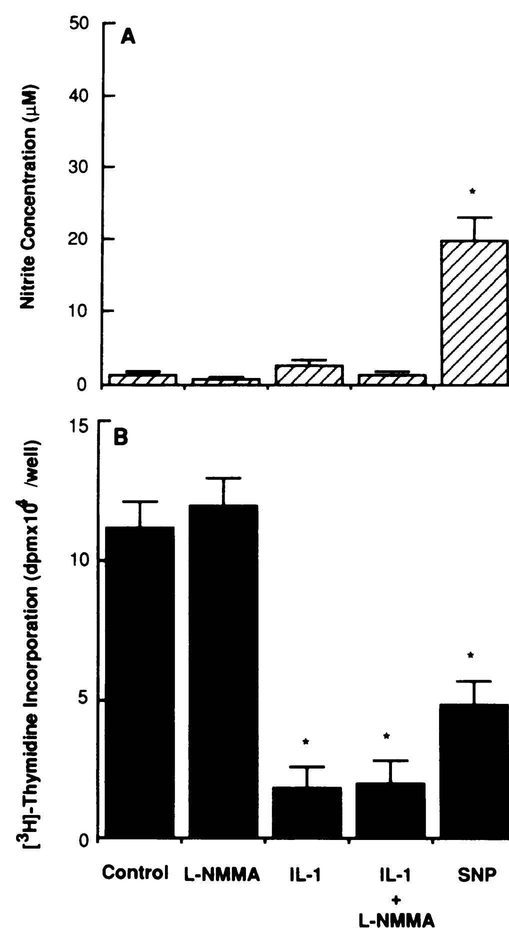

Figure 5. Effects of IL-1 and SNP on NO release and DNA synthesis in EC alone. Confluent EC cultured on 24-well plates were incubated for 24 h with serum-free medium containing SNP or IL-1 in the presence or absence of L-NMMA. EC were labeled with [^{3}H]thymidine during the last 4-h period of the incubation. Nitrite concentration (*A*) and DNA synthesis (*B*) were determined as described in the text. Values are mean±SEM of three individual experiments containing three replicates in each experiment. $*P < 0.05$, significantly different from control.

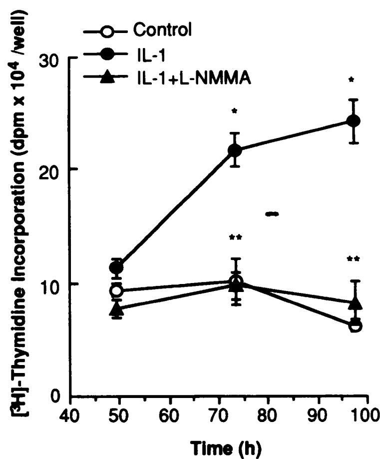

Figure 6. Effects of conditioned medium from VSMC incubated with IL-1 on DNA synthesis in EC. Quiescent VSMC were cultured in DME containing vehicle or 1 nM IL-1 in the presence or absence of 3 mM L-NMMA for the times indicated. After the incubation, conditioned medium was collected aseptically and stored at −20°C until assay. Conditioned medium was then added to confluent EC and incubated for 20 h. EC were then pulse-labeled with [^{3}H]thymidine for a further 4 h. [^{3}H]Thymidine incorporation in EC was measured as described in the text. Values are mean±SEM of two individual experiments containing four replicates in each experiment. *$P < 0.05$, significantly different from control. **$P < 0.05$, significantly different from cells treated with IL-1 alone.

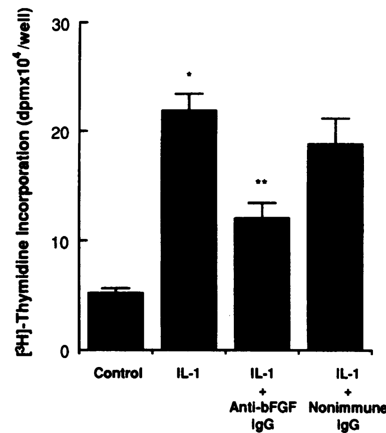

Figure 7. Effects of anti-bFGF IgG on the stimulation of DNA synthesis of EC induced by conditioned medium from VSMC incubated with IL-1. Conditioned medium from VSMC incubated for 96 h with IL-1 in the presence or absence of L-NMMA was collected as described in the legend to Fig. 6. Conditioned medium was then added to confluent EC in the presence or absence of anti-bFGF IgG (10 μg/ml) or nonimmune IgG (10 μg/ml) and incubated for 20 h. EC were then pulse-labeled with [^{3}H]thymidine for a further 4 h. [^{3}H]-Thymidine incorporation in EC was measured as described in the text. Values are mean±SEM of three individual experiments containing four replicates in each experiment. *$P < 0.05$, significantly different from control. **$P < 0.05$, significantly different from cells treated with IL-1 alone.

conditioned medium from VSMC incubated for 96 h with IL-1, whereas nonimmune IgG had no effect (Fig. 7).

Immunolocalization of bFGF in VSMC. Next, we examined the subcellular immunolocalization of bFGF in VSMC by ABC method. As shown in Fig. 8, immunocytochemical staining with anti-bFGF IgG revealed intense and diffuse staining throughout the cytoplasm of VSMC.

IL-1 stimulates bFGF release from VSMC. Fig. 9, *A* and *B*, shows the kinetics of changes of bFGF concentration and LDH activity in the medium after the stimulation of VSMC with SNP or IL-1 in the presence or absence of L-NMMA. IL-1 significantly enhanced the release of bFGF and LDH in the medium time-dependently from 72 to 96 h after its stimulation of VSMC. The degree of change of bFGF release was correlated with that of LDH release in the conditioned medium. Coincubation with L-NMMA completely inhibited bFGF and LDH release induced by IL-1. Furthermore, incubation with SNP also enhanced the bFGF and LDH release in the conditioned medium time-dependently.

Discussion

In this study we demonstrated that prolonged incubation with IL-1 induced both high levels of NO production and cytotoxicity in VSMC. L-NMMA, an inhibitor of NO synthesis, inhibited both NO production and cytotoxicity induced by IL-1. Furthermore, L-NMMA–induced inhibition was partially but significantly blocked by coincubation with a 10-fold excess of L-Arg, suggesting that NO mediates IL-1–induced cytotoxicity in VSMC.

High levels of NO released from macrophages may not only be toxic to microbes and malignant cells but may also damage healthy tissue since a high level of NO impairs the function of mitochondrial and other FeS-containing enzymes which are important in the regulation of cell function and viability (26–28). Recently, Ellman et al. (29) also reported that NO mediates IL-1–induced cytotoxicity in rat ovary. Furthermore, NO is also suggested to mediate macrophage death by apoptosis (30).

IL-1 is also known to stimulate production of prostaglandin E_2, which inhibits DNA synthesis in VSMC (31). Prostaglandins have been recently reported to induce apoptosis in primary cultures of sheep ovarian surface EC but not in a human cancer cell line of epithelial origin (32). We have recently shown that NO partially mediates IL-1–induced prostaglandin E_2 production in VSMC (33). However, coincubation with indomethacin, a cyclooxygenase inhibitor, did not prevent the cytotoxicity induced by prolonged incubation with IL-1.

On the other hand, short incubation for 24 h with IL-1 stimulated [^{3}H]thymidine incorporation in VSMC. This result is consistent with previous reports (34, 35). Furthermore, prolonged incubation with IL-1 did not inhibit but rather stimulated [^{3}H]thymidine incorporation when NO synthesis was inhibited by L-NMMA, suggesting that IL-1 exerts both stimulatory and inhibitory signals for DNA synthesis in VSMC (35, 36). The mitogenic activity of IL-1 for VSMC may be mediated by induction of platelet-derived growth factor A-chain gene as reported previously (35).

Since IL-1 mRNA expression is enhanced in a human atherosclerotic plaque and IL-1 is produced by foam cells, VSMC, and microvascular endothelium, IL-1 is thought to play important roles in the pathogenesis of atherosclerosis (37, 38). Although the emphasis has been placed on the growth stimulatory effects of IL-1 on VSMC in vitro (34, 35), not only proliferation of VSMC but also cell necrosis are found in atherosclerotic lesions in vivo. In other words, increased cell turnover (vascular remodeling) is observed in these lesions. In this study, we demonstrated that IL-1 induces biphasic stimulation of [^{3}H]-thymidine incorporation in VSMC after the induction of NO synthesis. Furthermore, prolonged incubation of VSMC with IL-1 induces cytotoxicity through production of high levels of NO in VSMC themselves. Thus, IL-1 could induce both cell proliferation and cell damage in VSMC and then could stimulate vascular remodeling in atherosclerotic plaques in vivo.

In the coculture system of VSMC and EC without direct

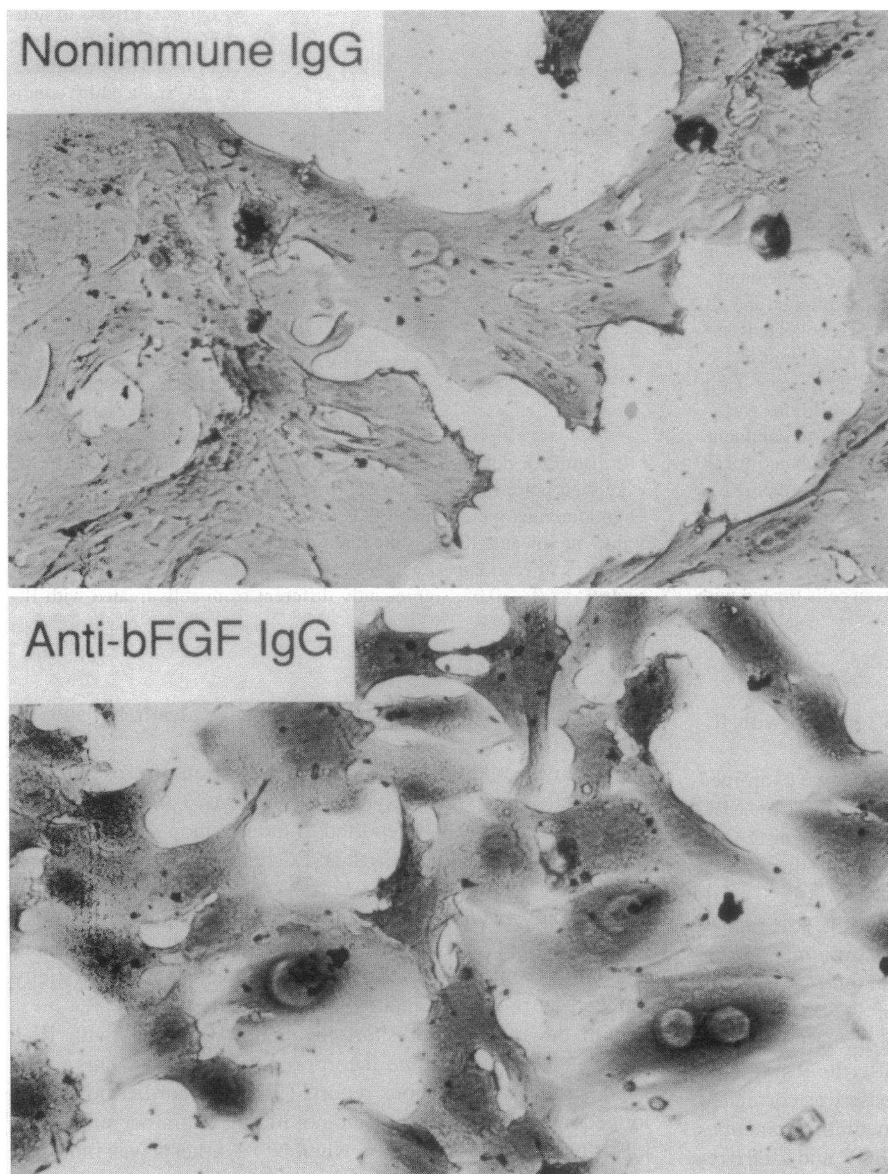

Figure 8. Localization of bFGF in VSMC. The concentration of primary antibody used was 10 μg/ml. VSMC were immunostained with anti-bFGF IgG or control IgG using ABC method as described in the text. Original magnification ×200.

cell contact, cocultured EC did not affect NO release and DNA synthesis induced by IL-1 in VSMC. On the other hand, cocultured VSMC affected the response to IL-1 in EC. Although IL-1 inhibited DNA synthesis in EC alone, DNA synthesis in EC was significantly stimulated by IL-1 when EC were cocultured with VSMC. Coincubation with L-NMMA completely inhibited the IL-1–induced stimulation of DNA synthesis in EC cocultured with VSMC, suggesting that NO mediates EC proliferation (39). However, SNP, a NO donor, did not stimulate but inhibited DNA synthesis in EC alone. Furthermore, conditioned medium from VSMC incubated with IL-1 for 72 and 96 h but not for 48 h stimulated EC proliferation. Therefore, it is unlikely that NO, an unstable molecule, directly stimulates DNA synthesis in cocultured EC. However, NO plays a key role in inducing the stimulation of DNA synthesis in cocultured EC, since conditioned medium from VSMC incubated with both IL-1 and L-NMMA lost the stimulatory activity on DNA synthesis in EC cocultured with VSMC.

Immunocytochemical staining with anti-bFGF IgG demonstrated intense and diffuse staining throughout the cytoplasm of VSMC. Furthermore, bFGF was detected in the conditioned medium from VSMC incubated for 72 and 96 h with IL-1, suggesting that bFGF may be a factor responsible for stimulation of DNA synthesis in cocultured EC induced by IL-1 or SNP. However, coincubation with anti-bFGF IgG partially neutralized the stimulation of DNA synthesis in EC induced by conditioned medium from IL-1–treated VSMC. Therefore, other EC growth factors secreted from VSMC such as vascular endothelial growth factor, which is a recently characterized endothelial-specific mitogen (40, 41), may also be involved in the stimulation of DNA synthesis in EC induced by conditioned medium as well as bFGF.

bFGF is known to be a potent EC and VSMC mitogen (42, 43) and is synthesized by three cell types associated with atherosclerosis: EC (44), macrophages (45), and VSMC (46). Brogi et al. (47) recently demonstrated that immunolocalization revealed abundant bFGF protein in control vessels but little in plaques, although levels of mRNA did not differ among control

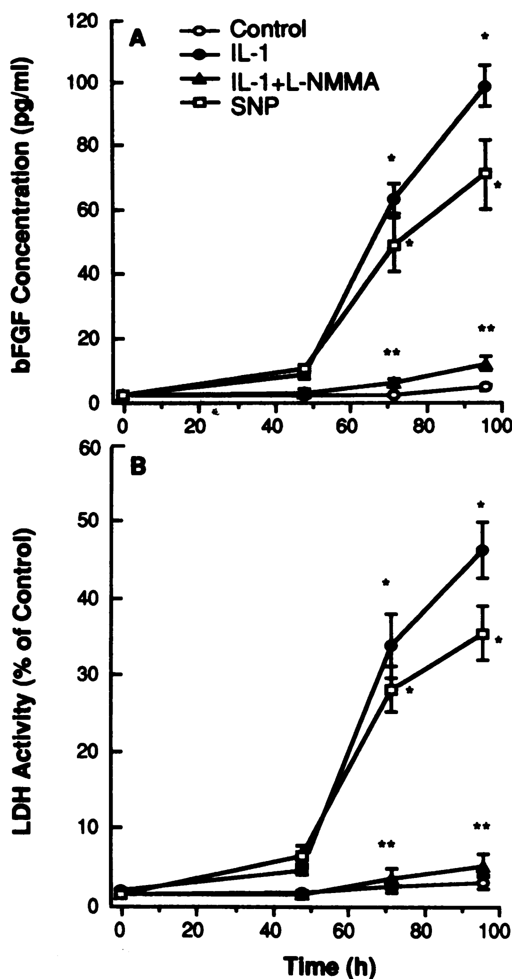

Figure 9. Kinetics of bFGF and LDH release in conditioned medium from VSMC incubated with IL-1. Conditioned medium from VSMC incubated with SNP or IL-1 in the presence or absence of L-NMMA was obtained as described in the legend to Fig. 6. LDH activity (*A*) and concentration of bFGF (*B*) in the conditioned medium were determined as described in the text. Values are mean±SEM of four individual experiments containing two replicates in each experiment. $*P < 0.05$, significantly different from control. $**P < 0.05$, significantly different from cells treated with IL-1 alone.

and plaque specimens. Furthermore, Edelman et al. (48) also reported that exogenous administration of bFGF enhances the coupling of intimal hyperplasia and proliferation of vasa vasorum in injured rat arteries. However, biosynthetic studies indicate that bFGF is a cellular rather than a secreted protein and may be released from cells after injury (49). Linder et al. (8, 10) recently reported that proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against bFGF and by heparin.

In this study, the degree of bFGF release into the medium was correlated with the degree of cytotoxicity in VSMC, suggesting that damaged VSMC release bFGF into the medium. Coincubation with L-NMMA completely inhibited both bFGF release and cytotoxicity in VSMC induced by prolonged incubation with IL-1. Furthermore, SNP stimulated bFGF release in the medium from VSMC. Taken together, these findings suggest that prolonged incubation of VSMC with IL-1 induces high levels of NO which mediate cytotoxicity in VSMC, and that damaged VSMC then release bFGF which stimulates adjacent EC proliferation. Since bFGF is a strong angiogenesis-inducing factor, these findings may be important in the mechanism of neovascularization in atherosclerotic plaques (50). Neovascularization of atherosclerotic plaques may have significant clinical consequences (51). A high proportion of patients dying of myocardial infarction shows evidence of intraplaque hemorrhage, presumably from small vessels in the plaque (52, 53).

In cocultured VSMC, prolonged incubation with IL-1 induced high levels of NO release to the same degree as in VSMC alone. High levels of NO induced by IL-1 or SNP inhibited DNA synthesis in cocultured VSMC. In contrast, a high level of NO did not inhibit but rather stimulated DNA synthesis in EC cocultured with VSMC, although it inhibited DNA synthesis in EC alone. These results suggest that EC may become resistant to the cytotoxic effects of high levels of NO when they are cocultured with VSMC. Since bFGF inhibits EC death by apoptosis induced by serum deprivation (54), bFGF may be involved in the mechanism of NO resistance in EC. However, our preliminary experiments revealed that coincubation with bFGF significantly prevented the cytotoxicity induced by SNP not only in EC but also in VSMC (data not shown), suggesting that bFGF can prevent NO-induced cytotoxicity in both EC and VSMC. On the other hand, bFGF was detected in the conditioned medium from VSMC when VSMC were damaged by high levels of NO. Therefore, cocultured EC could be damaged by high levels of NO as well as VSMC before bFGF is released. These results suggest that other VSMC-derived factors released from intact VSMC may be involved in NO resistance as well as bFGF in cocultured EC. Vascular endothelial growth factor, a VSMC-derived factor, which is a mitogen for EC but not for VSMC (40, 41) and enhances the action of bFGF (55), could be a candidate for this VSMC-derived factor. However, further studies are needed to clarify these points.

In conclusion, our results indicate that high levels of NO released from VSMC mediate cytotoxicity in VSMC but not in adjacent EC. Damaged VSMC release bFGF which stimulates adjacent EC proliferation. Thus, VSMC damage induced by high levels of NO may be an important trigger of neovascularization in atherosclerotic plaques, and NO released from VSMC may be an important signal in the progression of atherosclerosis through cellular interactions between EC and VSMC.

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