Vascular Endothelial Cell Regulation of Extracellular Matrix Collagen
Role of Nitric Oxide
Paul R. Myers, Miles A. Tanner

Abstract—Endothelium-derived products have been implicated in the regulation of vascular wall structure through their effects on extracellular matrix metabolism. The purpose of this study was to further understand the paracrine mechanisms underlying endothelial cell regulation of extracellular matrix metabolism by testing the hypothesis that endothelium-derived nitric oxide decreases the concentration of soluble collagens derived from vascular smooth muscle cells (VSMCs). Porcine coronary endothelium and VSMCs were grown under a coculture configuration to assess the paracrine effects of nitric oxide produced by the endothelium on VSMC collagen types I and III. Endogenous endothelial cell nitric oxide production was blocked with N⁶-nitro-L-arginine methyl ester. Collagen type I and type III were quantitatively measured using an enzyme-linked immunosorbent assay method. The endothelium elicited a time-dependent increase in the concentration of soluble VSMC-derived collagen type I; in contrast, collagen type III was decreased. After inhibition of nitric oxide production, there was a marked increase in both collagen types I and III concentration. These results demonstrated that endothelium-derived nitric oxide differentially alters collagen subtypes produced by VSMCs. The data support the hypothesis that nitric oxide functions via a paracrine mechanism to decrease VSMC collagen types I and III concentration, a finding consistent with an integral role for the endothelium in modulating extracellular matrix metabolism. (Arterioscler Thromb Vasc Biol. 1998;18:717-722.)

Key Words: extracellular matrix ■ nitric oxide ■ collagen ■ endothelium

Nitric oxide is an endothelium-derived compound that exerts multiple effects on the vasculature through its paracrine actions on the vessel wall, as well as on formed blood elements. The vasomotor actions of NO are well characterized (for review, see Reference 1). Nitric oxide also is a potent inhibitor of platelet aggregation,2,3 an antimetabolite,4-7 an inhibitor of cell migration,8 and an immunomodulator.9-11 Alterations and/or abnormalities in NO metabolism have been implicated in a variety of diseases, especially atherosclerosis12-15 and, more recently, restenosis.8,17 The multiple actions of NO and its role in vascular wall disease are consistent with an important role in the metabolism of the extracellular matrix.

The vascular wall extracellular matrix comprises the bulk of the interstitium of the media and is composed of a complex mixture of extracellular matrix proteins, including collagens.18 The fibrillar collagens are the most abundant collagens, with subtypes I, III, and IV being most common.18,19 Atherosclerosis and restenosis are two disease processes relevant to coronary artery vascular biology that are directly related to extracellular matrix collagen synthesis and degradation, primarily because the hallmark of the disease is mechanical obstruction of coronary blood flow secondary to medial and intimal hyperplasia. The importance of a functionally intact endothelium in atherosclerosis is firmly established.20 Less well characterized, however, is the role of the endothelium in extracellular matrix metabolism, despite the observation that the extracellular matrix comprises the bulk of the media in health and the bulk of the abnormal neointima during disease.

The purpose of the present study was to employ coronary endothelium and coronary VSMCs in a coculture configuration to more closely mimic a paracrine environment to test the hypothesis that endogenously derived endothelial cell NO directly affects collagen subtype concentrations. Prior research has demonstrated that NO donors alter collagen metabolism when added directly to VSMCs21,22; however, less is known with regard to the role of endogenously derived NO in vascular wall extracellular matrix metabolism. Since previous work has established that NO is produced by an endothelial constitutive enzyme, the experimental design employed a NOS antagonist to block endogenous synthesis of NO.

Methods

Tissue Culture
Primary cultures of porcine coronary artery endothelial cells were established by lightly touching the luminal surface of a freshly harvested epicardial coronary artery with a sterile Q-tip and dipping the cotton in filtered endothelium-conditioned growth medium (me-
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1-mm² pieces. Each piece of tissue was plated directly into a 3.8-cm² dish 199 containing Earle’s salts and 0.15 g neo mycin) supplemented with 20% (vol/vol) FBS (Hyclone). The dishes were plated into a 95% air/5% CO₂–atmosphere incubator at 37°C and allowed to grow until established colonies became apparent. Cells were then lightly trypsinized with 0.05% trypsin (0.5 mmol/L EDTA), and single cells were identified under a microscope and transferred to 12-well plates with a sterile glass pipette mounted on a micromanipulator. Single cell clones were grown to confluence and identified as endothelium using morphology, factor VIII antigen staining, presence of acetylated LDL receptors, and NO production. The resulting monocultures were subcultured and referred to as first-passage cells. The medium was changed every 2 to 3 days. Experiments were performed on fourth- to eighth-passage cells. The medium was changed every 2 to 3 days. The medium was changed every 2 to 3 days. The medium was changed every 2 to 3 days.

Coculture Protocol

Endothelium

Identical numbers of cells (density of 1 to 3×10⁶ cells per insert) were plated onto Falcon inserts (catalog No. 3091, polystyrene, pore size 3 µm) in medium 199 with Earle’s salts and 15% FBS. Inserts were allowed to incubate under the same conditions (95% air/5% CO₂) for 24 to 48 hours. The endothelial cells were washed with warm PBS ×2, then placed in 0.4% FBS for 24 hours. The wash routine was repeated, at which time the medium was replaced with medium 199 with Earle’s salts containing 0.4% FBS.

Vascular Smooth Muscle

Identical numbers of cells were plated onto Falcon six-well dishes that served as “companion plates” (catalog No. 3502; density of 1 to 3×10⁵ cell per well) to the inserts on which endothelium was grown. Cells were grown in medium 199 with Earle’s salts and 15% FBS, allowed to incubate under the same conditions 24 to 48 hours, then washed with warm PBS ×2, and placed into 2 mL medium supplemented with 0.4% FBS for another 24 hours.

Coculture

On the day of an experiment, growth medium was removed from the wells containing VSMCs and also the inserts containing endothelium, the insert was carefully lowered into the companion well and medium (supplemented with 0.4% FBS) was then added to the assembly, ensuring the absence of air bubbles. Drugs (50 µmol/L L-NAME and 5 µmol/L indomethacin, Sigma Chemical Company) were added to the insert, and carrier was added to the inserts of control wells. All experimental conditions were prepared in triplicate.

Collagen Type I and III Assay

An ELISA, originally described by Engvall,2 was modified and used to directly measure soluble collagen in the growth medium. Soluble collagen was assayed because previous studies demonstrated fibrillar collagen synthesized in cell culture is soluble3 and distributed primarily in the growth medium.3 Serial dilutions of antigen (human collagen type I and type III: Southern Biotechnology Associates) were prepared with SBB, pH 9.6. One hundred microliters of each dilution was added to a well of a 96-well modified flat-bottom polystyrene microtiter plate (Corning, model 25805 to 96) for generating a standard calibration curve. One hundred microliters of the SBB was used to determine background. One hundred–microliter samples of growth medium with the amount of collagen to be determined were added to separate wells on the same plate. One hundred microliters of medium 199 supplemented with 0.4% FBS incubated in blank companion wells containing an insert with no cells served as control. The 96-well plate was incubated at 4°C for 24 hours in a humidified atmosphere. Excess antigen was removed by washing three times with 100 mmol/L BBS, pH 8.0, with 0.05% Tween 20 (BBS-T, pH 8.0) in an automated microplate washer. Nonspecific protein binding sites were blocked by completely filling the wells with 300 µL of 0.1% bovine serum albumin (pH 8.0) in BBS and incubating at room temperature (25°C) with gentle shaking for 60 minutes. Excess albumin was removed by washing three times with BBS-T. One hundred microliters of appropriate dilutions of primary antibody against collagen type I (goat anti-human and bovine type I collagen; <10% cross-reactivity with types I, II, III, IV, and VI; Southern Biotechnology Associates) or against collagen type III (goat anti–type III collagen affinity purified antibody; <10% cross-reactivity with types I, II, IV, and VI; Southern Biotechnology Associates) was added to each well and incubated for 60 minutes at room temperature with gentle shaking. Excess primary antibody was removed by washing five times with BBS-T. One hundred microliters of appropriate dilutions of biotin-conjugated secondary antibody (goat anti-rabbit IgG H&L, biotin-conjugated antibody, BioDesign International; or rabbit anti-goat IgG H&L, biotin-labeled antibody, Southern Biotechnology Associates) was added to each well and incubated at room temperature for 60 minutes with gentle shaking. Excess antibody was removed by washing five times with BBS-T. One hundred microliters of a 1:8000 dilution of streptavidin hors eradish peroxidase–labeled (Southern Biotechnology Associates) solution was added to each well and incubated for 60 minutes at room temperature with gentle shaking. Excess streptavidin was removed by washing five times with BBS-T. One hundred microliters of freshly prepared substrate for horseradish peroxidase (ABTS, Sigma) was added to each well. Reaction products were quantified by reading of the microplates at 405 nm in a microplate autoreader spectrophotometer after 30 minutes. The optical density reading of control standards (100 µL of SBB) was subtracted from optical density readings of each test standard and hence calibration curves for the determination of collagen types I and III by ELISA were obtained. Optical density reading of control from culture media (100 µL of medium 199/0.4% FBS) was subtracted from optical density readings from each unknown. The concentration of collagen type I and collagen type III in the unknown was determined by using the linear portion of the standard curve. The standards, appropriate controls, and each unknown were prepared in duplicate and the values averaged.

Determination of ELISA Conditions for Collagen Type I and Type III

For collagen type I, microtiter plates were coated with serial dilutions of standard collagen type I (5000 to 11.5 ng/mL) and incubated with serial dilutions of primary antibody (goat anti-human

**Selected Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BBS</td>
<td>borate-buffered saline</td>
</tr>
<tr>
<td>BBS-T</td>
<td>BBS with Tween 20</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>H&amp;L</td>
<td>heavy and light chain</td>
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<tr>
<td>L-NAME</td>
<td>L-arginine methyl ester</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>SBB</td>
<td>sodium bicarbonate/disodium carbonate buffer</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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type I, 1:1600) and secondary antibody (rabbit anti-goat IgG H&L biotin-conjugated, 1:50 000). Streptavidin horseradish peroxidase and ABTS incubations were done as described above to determine the optimal primary and secondary antibody dilutions to yield linearity and a good color reaction for the appropriate concentration range of collagen type I.

For collagen type III, microtiter plates were coated with serial dilutions of standard collagen (2500 to 1.0 ng/mL) and incubated with serial dilutions of primary antibody (goat anti-human type III collagen affinity purified antibody, 1:100) and secondary antibody (rabbit anti-goat IgG H&L, biotin-labeled, 1:10 000). Streptavidin horseradish peroxidase and ABTS incubations were done as described above to determine the optimal primary and secondary antibody dilutions to yield linearity and a good color reaction for the concentration range of collagen type III.

**Determination of Protein Synthesis and Cell Counts**

Cellular protein derived from both coronary artery VSMCs and endothelium was determined according to Bradford (BioRad) with bovine serum albumin as a standard. Cell protein was solubilized with 0.1N NaOH. Cells were manually counted in triplicate using a hemocytometer and the manufacturer’s instructions.

**Experimental Protocol**

Cocultures were performed according to the paradigm outlined in the diagram below for 6, 12, 24, 36, or 48 hours’ duration to determine the optimal time period to elicit coculture effects on collagen I and collagen III concentration in the growth medium. Since maximal effects were seen at 24 to 36 hours, antagonist studies were carried out at 36 hours’ coculture.

L-NAME and/or indomethacin (to eliminate any potential confounding effects of cyclooxygenase products) were added to the coculture assembly and the medium and cells were harvested separately at the end of 36 hours. Cellular protein was determined on each sample and was taken to reflect the effects of coculture on cell division.

**Data Analysis**

The effects of coculture on either collagen I or collagen III concentration was determined by A divided by B (see diagram). Time-course data were obtained as nanograms collagen type I or type III per microgram protein and expressed as the percent change relative to VSMC cultures without endothelium. For any one number (n), the average was calculated from measurements obtained from triplicate cultures. Separate experiments on different days comprised a sample number.

The effects of NOS inhibition (L-NAME) were expressed as both raw data (nanograms per microgram protein) and the mean of the percent change in collagen concentration elicited by the drug relative to cocultures without drug ± SEM (see diagram): [(C−A)/A] for coculture and [(D−B)/B] to ascertain the direct effects of drug on VSMCs alone.

The effects of coculture on VSMC cell protein (harvested from the well) were expressed as percentage determined as VSMC protein in the presence of endothelium divided by VSMCs in the absence of endothelium.

The effects of coculture on endothelial cell protein (harvested from the insert) were determined similarly. Statistical significance between the amounts of collagen at different time points or the significance of the effects of NOS inhibition in coculture versus VSMCs alone was established using the two-tailed, unpaired Student’s t test. P<.05 was considered statistically significant. Samples were always done in triplicate and n represents independent experiments done on separate dates. When multiple drug conditions were analyzed for statistical difference, data were compared by analysis of variance with a Bonferroni correction for multiple comparisons.

**Results**

**Effects of Coculture on VSMCs and Endothelial Cell Protein Concentrations**

When grown in the coculture configuration in low-serum medium, the presence of endothelial cells resulted in a significant decrease in VSMC protein compared with VSMCs grown in low-serum medium without endothelium (Fig 1). In contrast, the VSMCs in coculture had little effect on endothelial cell protein (Fig 1).

Separate experiments were done in a noncoculture configuration to determine the direct effects of low-serum (0.4%) medium on both VSMC and endothelial cell number, cell protein, and cell viability. Results were compared with growth in medium supplemented with 15% serum. Compared with VSMCs or endothelium grown in normal medium, there was a significant decrease in total cell protein and cell number. There was no significant difference in cell viability with either VSMCs or endothelium when cells were grown in low-serum medium (Table). The decrease in endothelial cell protein was accompanied by a proportional decrease in cell number (41.6±14.98% reduction in protein; 33.5±5.5% reduction in cell count relative to cells grown in 15% serum). The decrease in VSMC protein was accompanied by a proportionally larger decrease in cell number (43.8±4.2% reduction in total protein; 63±1.9% reduction in VSMC count relative to cells grown in 15% serum). These data suggest that in low-serum medium there is decreased cell division, with no change in cell viability or survival.
**Time Course for Coculture Response**

The collagen I and collagen III concentrations in the growth medium of cocultures incubated for 6, 12, 24, and 36 hours were quantitatively determined. At 24 and 36 hours, coculture with endothelium resulted in a significant time-dependent increase in collagen I compared with VSMCs without endothelium (Fig 2A).

In contrast to the findings with collagen I, at 12, 24, and 36 hours, the presence of endothelium resulted in a significant time-dependent decrease in collagen III concentration compared with cultures of VSMCs alone (Fig 2B).

**Effects of Endothelium Coculture on VSMC Collagen I Concentrations**

**Collagen I**

Under basal, coculture conditions without any antagonists, the growth medium concentration of VSMC-derived collagen I was significantly increased compared with that measured in the absence of endothelium (Fig 3, inset). After blockade of endogenously produced NO with L-NAME (50 μmol/L) and in the presence of indomethacin, there was a significant augmentation of the endothelial cell stimulatory effect on collagen I concentration compared with that observed in cocultures of endothelium and VSMCs without drug (Fig 3 inset compared with Fig 3, L-NAME + indomethacin). Identical experiments were done with N^\text{G}-nitro-D-arginine methyl ester, and the effects observed after L-NAME were not seen, consistent with the stereoisomer-specific effect of L-NAME.

**Collagen III**

Under basal, coculture conditions without any antagonists, the growth medium concentration of collagen III was significantly decreased when endothelium was present for 36 hours (Fig 4, inset).

In cultures of VSMCs alone without endothelium, incubation with L-NAME and indomethacin resulted in a decrease in collagen I concentration; however, this result was not statistically significantly different from control VSMC cultures without endothelium (P > .05).

**Effects of Low Serum on Cell Protein, Cell Count, and Cell Viability (Percent Change)**

<table>
<thead>
<tr>
<th></th>
<th>Cell Protein</th>
<th>Cell Count</th>
<th>15% FCS</th>
<th>0.4% FCS</th>
<th>Viability in</th>
<th>Viability in</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSMCs</td>
<td>-43.8±4.2</td>
<td>-63±1.9</td>
<td>-41.6±14.98</td>
<td>-33.5±5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>97.35±0.1</td>
<td>95.6±0.6</td>
<td>95.4±0.4</td>
<td>93.2±0.1</td>
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Cells were grown in either 15% FCS or 0.4% FCS for 36 hours in six-well plates. At the end of the time period, cells were counted and the total cell protein was determined. Viability was determined by using trypan blue dye exclusion. Data are expressed as percent reduction in cell protein or cell numbers obtained from cells grown in 15% FCS (n = 4).
endothelium and VSMCs without drug. L-NAME resulted in a significant increase in collagen III concentration in contrast to the decrease seen without drug (Fig 4). However, there was no significant difference between cocultures of endothelium and VSMCs compared with VSMCs alone treated with L-NAME and indomethacin.

In cultures of VSMCs alone without endothelium, incubation with L-NAME and indomethacin trended toward a further decrease in collagen III that did not reach statistical significance when compared with control cultures of VSMCs alone.

**Discussion**

The results obtained from these experiments are consistent with the original hypothesis that the endothelium elicits time-dependent changes in VSMC collagen metabolism through the paracrine actions of endogenously derived factors. We used a coculture system to specifically study the contribution of endogenously produced NO to endothelial cell paracrine effects on collagen concentration. An important advantage of the coculture system is that it provided a primary source of agonist derived from an intact endothelial cell with functional, endogenous regulatory feedback mechanisms present. Endothelial cell factors were produced at a constant, basal rate in an environment that more closely mimics that found in vivo and avoided the variable concentrations seen with unstable NO donors that we have previously studied. The data suggest (1) the presence of endogenous endothelial cell regulators of extracellular matrix metabolism in the vascular wall and (2) a regulatory role for NO as a mechanism to influence vascular wall extracellular matrix metabolism.

The experimental design employed here depended on the presence of basal release of NO, which, via a paracrine mechanism, was hypothesized to alter VSMC collagen metabolism. We have previously measured basal release of NO, prostacyclin, and prostaglandin E\(_2\) from endothelium,\(^{27}\) as well as inhibition of NO production, with the concentration of L-NAME used in this study. Thus, we had prior evidence that L-NAME at the concentration used would abolish endogenous NO production. In an effort to decrease potential complications via the production of prostanoids, indomethacin was added to the coculture experiments as part of the experimental design. The increase in collagen I concentration after inhibition of NO production was observed only after cyclooxygenase inhibition, since L-NAME alone had no significant effect. This observation is consistent with complex interactions between cyclooxygenase and NOS. To uncover the effects of NO in this particular study, prostanoid production was inhibited. The exact mechanism underlying this observation is presently not known.

The mechanism(s) responsible for the net increase in collagen I or net decrease in collagen III in the coculture system elicited by the endothelium may involve complex interactions between several paracrine agents that affect both synthesis and breakdown of soluble fibrillar collagen. Since we measured collagen concentration, the data here do not distinguish between altered synthesis and/or breakdown. The alteration of endogenous NO production could result in altered breakdown of collagen I by increased or decreased production of metalloproteinases and/or decreased tissue inhibitor of metalloproteinase (TIMP) levels.

Our previous data indicated that cultured coronary VSMCs do not produce NO basally. Thus, the effects of NOS inhibition most likely reflect the effects of endothelium alone.

The observation that cocultures of endothelium and VSMCs increase VSMC-derived collagen I concentration and decrease VSMC-derived collagen III concentration in the growth medium concurs with previous reports from rat fibroblasts cocultured with bovine aortic endothelium.\(^{28}\) However, the current data provided new information complementing these observations because they (1) used endothel-
um and VSMCs from the same vasculature bed (ie, coronary), (2) implicate a mechanistic role for the endogenously derived endothelial paracrine agent NO in extracellular matrix metabolism, and (3) quantified the most abundant collagen subtypes (I and III) in the vascular wall. The ability of NO to modulate collagen metabolism has been previously reported in noncoronary tissue. Trachtman et al23 reversed the effect of γ-interferon and lipopolysaccharide on renal mesangial cell accumulation of matrix collagens by inhibiting NOS with L-NAME. Previous experiments have demonstrated that NO donors added directly to the VSMC growth medium decreased collagen I concentrations, but not collagen III concentrations.21 Kolpakov et al22 have also previously found that NO inhibits total protein and collagen synthesis in noncoronary VSMCs.

Coculture conditions were strictly controlled with regard to cell number and the coculture configuration. Identical numbers of cells were seeded for any particular experiment and grown in low-serum medium sufficient to maintain viability but arrest cell division. We have found that growth in no serum results in cell death and precludes long-term incubations such as the ones we used here. However, 0.4% serum did not alter viability significantly over the time courses used. Since all cells were grown under identical conditions, the decrease in VSMC protein during coculture with endothelium is consistent with antimitogenic effects of endothelium-derived factor(s). Although the identity of the exact factor(s) is unknown, this observation is in agreement with previously published data on the antimitogenic effects of NO.21

In summary, endogenously derived endothelial cell products, via a paracrine mechanism, can alter extracellular matrix metabolism. The coronary artery endothelium cocultured with coronary VSMCs resulted in a time-dependent increase in the concentration of collagen type I, but not collagen type III; instead, collagen type III decreased. After inhibition of NO production, there was a marked increase in collagen type III and a smaller increase in collagen type I concentrations. The data support an inhibitory role for NO on collagen type I and collagen type III production and suggest that diseases impairing endogenous production of NO may adversely affect an important regulatory mechanism underlying the control of vascular collagen metabolism.

Acknowledgments

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References

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