Interactions Between the Monocyte/Macrophage and the Vascular Smooth Muscle Cell

Stimulation of Mitogenesis by a Soluble Factor and of Prostanoid Synthesis by Cell–Cell Contact

Hanfang Zhang, Elizabeth C. Downs, Jenifer A. Lindsey, W. Bruce Davis, Ronald L. Whisler, and David G. Cornwell

The effect of soluble factors from the monocyte/macrophage (MØ) on cell proliferation and the functional effects of cell–cell contact on the arachidonic acid (AA) cascade were studied with vascular smooth muscle cells (SMCs). Peripheral blood MØs were isolated by adherence or in a Percoll gradient, and alveolar MØs were obtained by lavage. Conditioned medium (CM) was prepared by preincubating MØs with medium alone or by separating SMC and MØ cocultures by a membrane insert. Cell proliferation (image analysis) and 6-ketoprostaglandin F1α (6-keto-PGF1α, radioimmunoassay) were measured in SMCs. Labeled prostanoids and other eicosanoid metabolites were isolated by high-performance liquid chromatography from SMCs prelabeled with 14C-AA. MØs did not synthesize 6-keto-PGF1α. The CM enhanced proliferation but did not stimulate 6-keto-PGF1α synthesis in SMCs. However, cell–cell contact in cocultures of SMCs with the same concentration of MØs used to generate CM resulted in increased 6-keto-PGF1α synthesis by SMCs. Since the stimulatory effect of cell contact was not blocked by butylated hydroxytoluene, it could not be attributed to an oxidative burst from MØs. Functional studies showed that the stimulatory effect of cell contact was enhanced by exogenous free AA and by endogenous AA release through A23187. Release of total radioactivity from prelabeled SMCs was enhanced by cell contact, and this effect was blocked by indomethacin (IM). Cell contact did not increase the release of free AA from prelabeled SMCs, even in the presence of IM. Finally, cell contact only stimulated the formation of prostanoids (IM-sensitive eicosanoid metabolites) from prelabeled SMCs. Lipooxygenase and other products of AA were not formed through cell–cell contact. These data showed that MØs express a soluble factor that enhances SMC proliferation without affecting prostanoid synthesis. Subsequent cell contact between SMCs and MØs stimulates prostanoid synthesis, which may possibly serve as a local and focal homeostatic mechanism for the regulation of uncontrolled SMC proliferation in atherogenesis.


KEY WORDS • smooth muscle cells • monocytes • proliferation • arachidonic acid cascade • cell–cell contacts • prostanoids

The recruitment of peripheral blood monocytes (MØs) is an early event in the progression of atherosclerosis, and the foam cell that is characteristic of the atherosclerotic lesion is derived from MØs. Atherosclerosis also involves smooth muscle cell (SMC) differentiation and proliferation, processes that are regulated in part by soluble factors from MØs and eicosanoid metabolites from both MØs and

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SMCs express a soluble growth factor for rapidly dividing SMCs that is heat labile and stimulated by bacterial lipopolysaccharide (LPS). The soluble growth factor is not interleukin-1 (IL-1), but the factor has platelet-derived growth factor (PDGF)-like activity that may be expressed through the action of IL-1 in inducing the PDGF-AA chain gene.

Prostanoid synthesis is higher in proliferating SMCs and it may be possible that the formation of prostanoids such as prostacyclin (PGI2) regulates SMC differentiation and proliferation. Peripheral blood and alveolar MØs do not synthesize PGI2, but MØs and, in particular, activated MØs elaborate polypeptide growth factors that stimulate prostanoid synthesis in several cell lines either through the expression of cyclooxygenase activity or phospholipase A2 activity. These observations suggest that soluble MØ factors are capable of both promoting SMC proliferation and inhibiting SMC proliferation by stimulating prostanoid synthesis. The potentially concurrent and seemingly paradoxical effects of soluble

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MØ factors on mitogenesis and the inhibition of mitogenesis are difficult to separate in the biology of the cell wall. The effects may be concentration dependent, or there may be alternative mechanisms that permit the separation of stimulatory and inhibitory effects on mitogenesis. For example, cell–cell contact between different cell types in the vessel wall is important in regulating cell proliferation, and SMCs leads to the formation of prostanoid but not lipoxigenase or other products of the arachidonic acid (AA) cascade. We suggest that the initial stimulatory effect of a soluble MØ factor on SMC proliferation is subsequently regulated by a process in which cell–cell contact between proliferating SMCs and recruited MØs generates prostanoids that function as agents for the local and focal homeostatic control of SMC proliferation in the vessel wall during atherogenesis.

Methods

Peripheral Blood Monocytes

Peripheral blood mononuclear cells were obtained by centrifugation of blood from normal human volunteers over Ficoll-Hypaque gradients. Peripheral blood mononuclear cells were rinsed twice in Seligmann’s balanced salt solution containing 2% human albumin and resuspended at 10^7 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum. Then 5 ml of the cell suspension was added to a 100-mm-diameter plastic Petri dish (No. 25020, Corning Glassworks, Corning, N.Y.) and incubated at 37°C in 95% air/5% CO2. Nonadherent cells were removed by rinsing with Seligmann’s balanced salt solution/2% albumin, and adherent MØs were harvested with a rubber policeman. The adherent MØs were more than 90% pure by immunofluorescence analysis, were functionally intact as assessed by chemotaxis and phagocytosis, demonstrated 90% or more esterase activity, and showed greater than 90% viability by trypan blue exclusion. In several experiments, adherent MØs were suspended in cloning medium (see “Smooth Muscle Cells” section in “Methods”) or cloning medium containing 5 mM EDTA and incubated for 10 minutes to remove adherent platelets. In other experiments, adherent MØs were lysed by freezing and thawing followed by sonication for 1 minute. No intact cells were visible under the microscope.

Peripheral blood MØs were also isolated from peripheral blood mononuclear cells in a Percoll gradient. Peripheral blood mononuclear cells were resuspended at 10^7 cells/ml in RPMI 1640 supplemented with 10% fetal bovine serum, and MØs were separated on a discontinuous gradient prepared from a Percoll stock solution (Pharmacia Fine Chemicals, Uppsala, Sweden). The gradient layers contained 50.0%, 47.5%, 45.0%, 42.5%, and 37.5% Percoll stock solution diluted with RPMI 1640. The Percoll gradient was centrifuged at 400g for 40 minutes. Percoll MØs were harvested from the top layer and routinely contained 75–90% MØs as identified by a Wright-Giemsa stain.

Alveolar Macrophages

Human alveolar macrophages (an example of a tissue MØ) were obtained by bronchoalveolar lavage of non-smoking normal volunteers as previously described. Briefly, lidocaine spray (1%) was used to anesthetize the upper airway and larynx. The bronchoscope was inserted through the nose and the tip wedged in a distal portion of a right middle lobe bronchus and a bronchus in the lingula of the left upper lobe. Five 20-ml aliquots of 0.15 M NaCl were instilled into each lobe for a total of 200 ml. Recovered lavage fluid was strained with gauze and centrifuged (400g, 10 minutes) to separate the cells. A small aliquot of the lavage fluid was cytocentrifuged, and the pellets were examined microscopically with the use of Diff-Quik stain (AHS del Caribe, Aguada, Puerto Rico). A minimum of 300 cells was counted for each lavage sample, and the average differential count was MØs 84.8%, lymphocytes 13.3%, neutrophils 2.1%, and eosinophils 0.2%.

Conditioned Medium

Supernatants from MØ cultures were used as the source of CM. Peripheral blood MØs prepared either by adherence or Percoll gradient and alveolar MØs were suspended at a known cell density in experimental medium or cloning medium. The suspension was incubated for 24 hours at 37°C. The CM was removed from the culture flask and centrifuged at 600g to remove any nonadherent MØs. The CM was used immediately or stored at −20°C until use. Storage did not affect the properties of the CM. In some experiments, the CM was boiled for 10 minutes. In other experiments, MØs were suspended in medium containing 5 μg/ml LPS (Escherichia coli WEO27:B8, DIFCO Laboratories, Detroit, Mich.) and incubated for 24 hours to prepare 24-hour CM-LPS. In other experiments, MØs were placed in an insert (Millipore, Belmont, Mass.) containing a Milli-cell-HA membrane (0.4-μm pore size) and cocultured for 24 hours with SMCs seeded outside the insert to prevent cell–cell contact. The CM was incubated for different periods of time with SMCs in the presence or absence of 10 mM indomethacin (IM).

Smooth Muscle Cells

Primary cultures of SMCs were obtained from the dissected medial layer of guinea pig aortas obtained from prepubertal male animals under ether anesthesia. Three guinea pigs were used for each primary culture. The experimental details for the tissue culture procedure are provided elsewhere. SMGs were identified by their reactivity to antibodies against human umbilical artery F-actin, which have been shown to react specifically with muscle actin isoforms. The medium used for growing cells to confluency (designated growth medium) was prepared from 1× Eagle’s minimum essential medium containing Hanks’ salts and 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (GIBCO, Grand Island, N.Y.) supplemented with 50 μg/ml gentamicin sulfate, 2 mM glutamine, 1×
nonessential amino acids (Microbiological Associates, Walkersville, Md.), 1 mM sodium pyruvate, 1.3 mg/ml NaHCO₃, and 10% fetal bovine serum (Stereile Systems, Logan, Utah). The medium used for prostanoid studies with confluent SMCs (designated experimental medium) was prepared from growth medium supplemented with 1× essential amino acids, 1× essential vitamins, and additional fetal bovine serum (final concentration, 20%). The medium used for SMC proliferation (designated cloning medium) contained the same supplements as experimental medium but with only 10% fetal bovine serum.

In proliferation experiments, SMCs (passage level 3 and 3 days after confluence was achieved) were seeded at 80 cells/cm² in Falcon single-well (60×15-mm) plates containing 4 ml cloning medium. The same number of cells was seeded in control and treatment plates in each experimental series. Cells were allowed to attach for 18 hours and then treated by the addition of 1 ml cloning medium alone or CM from MØ cultures. In all cloning studies, cells were re-treated with identical solutions on day 5 of the incubation period. After an 8-day incubation, medium was removed for analysis, and cells were fixed with 3.7% phosphate-buffered formalin and then stained with filtered Giemsa. A relative cell count was obtained from the total cell area on the Falcon plate. Total cell area was measured by image analysis using an Optomax Visual Analysis System (Optomax, Wallis, N.H.). The relation between cell area and cell number was validated by optical microscopy and with a Coulter Counter.

Radioimmunoassay of 6-Ketoprostaglandin F₁α

Tissue cultures were incubated with medium alone, CM, A23187, or LPS. Tissue cultures were also cocultured with MØs added directly to the medium, MØs separated within an insert, or lysed MØs. 6-Ketoprostaglandin F₁α (6-keto-PGF₁α), the stable metabolite of prostaglandin E₂ (PGF₂α), was then measured by radioimmunoassay as previously described for SMCs.²⁰-⁴⁹ Prostanoid synthesis under all incubation conditions was blocked by 10 μM IM. Antibodies were kindly supplied by Dr. Lawrence Levine, Brandeis University, Waltham, Mass. The cross-activity of the 6-keto-PGF₁α antibody was prostaglandin (PG) E₂ 0.15%, PGD₂ 0.02%, PGE₁ 0.10%, and AA 0.05%. The 6-keto-PGF₁α antibody unlike the PGE₂ antibody did not cross-react with nonenzymatic oxidation products of AA.⁵¹ Data for immunoreactive materials are expressed as nanomoles per culture (mean±SEM).

Labeling of Smooth Muscle Cell Lipids and Measurement of Labeled Arachidonic Acid Metabolites

[¹⁴C]AA (1.0 Ci/mmol) and [¹⁴C]AA (54.5 mCi/mmol) were purchased from New England Nuclear, Boston, Mass., and SMC lipids were labeled as previously described. Other experiments (data not shown) showed that IL-1 in concentrations ranging from 0.01 to 1.0 unit/ml had little effect on SMC proliferation, confirming previous studies that reported that MØs express a soluble growth factor for proliferating SMCs that is different from Il-1.¹² Although CM enhanced SMC proliferation, it had no stimulatory effect on PG₁₂ synthesis in SMCs (Table 1). Any increase in the 6-keto-PGF₁α content of the medium by the addition of 0.5 volume ethanol, acidification with formic acid, and extraction with ethyl acetate.²⁰-⁵² Recoveries of radioactive prostanoids and free AA were consistently greater than 90% when these compounds were added to the culture medium. Labeled metabolites were separated and identified by high-performance liquid chromatography (HPLC) as previously described.²⁰-⁵² Fractions were eluted from an Ultrasphere-ODS column by mixtures of acetonitrile/aqueous phosphoric acid. Fractions were collected at 0.5-1.0-minute intervals, counted, and compared with the elution of labeled standard compounds.

Statistics

Results are reported as mean±SEM.

Results

Peripheral Blood Monocytes Express a Soluble Factor Enhancing Smooth Muscle Cell Proliferation That Does Not Stimulate PG₁₂ Synthesis in Rapidly Dividing Smooth Muscle Cells

A series of experiments was undertaken with proliferating SMCs (Table 1). As expected from other studies,⁶⁰-⁶¹ CM from peripheral blood MØs stimulated SMC proliferation. The effect was proportional to the preincubation time with MØs and medium (compare 6-hour CM, 12-hour CM, and 24-hour CM data), indicating that increasing amounts of growth factor were expressed during preincubation. The MØ growth factor was not expressed by platelets trapped in adherent MØs, since an EDTA wash designed to remove platelets⁵⁵ did not affect the growth-promoting properties of CM (compare 24-hour CM, 24-hour CM₄, and 24-hour CM₅ data). The MØ growth factor was heat labile (compare 24-hour CM with 24-hour, boiled CM data), and it is important to note that boiling did not affect the growth-promoting properties of the medium alone (compare data for medium versus boiled medium). The MØ growth factor was increased when LPS was added to the medium during the preincubation process (compare 24-hour CM with 24-hour, boiled CM-LPS data). This effect is complex and difficult to interpret. LPS alone stimulated the synthesis of large amounts of PG₁₂, which evidently inhibits SMC proliferation. The inhibitory effect of increased PG₁₂ with LPS is blocked when IM is included in the incubation medium, and LPS under this condition actually enhanced SMC proliferation (compare data for medium, medium+LPS, and medium+LPS+IM). LPS also stimulated MØs to express additional growth factor, since the addition of LPS during preincubation enhanced the proliferative response with CM (compare data for medium+LPS+IM with either 24-hour CM-LPS or 24-hour CM-LPS-IM). These data confirmed previous studies showing that MØs express a heat-labile growth factor that is stimulated by LPS.⁶⁰-⁶¹ Other experiments (data not shown) showed that IL-1 in concentrations ranging from 0.01 to 1.0 unit/ml had little effect on SMC proliferation, confirming previous studies that reported that MØs express a soluble growth factor for proliferating SMCs that is different from IL-1.¹²
enhanced clonal growth with SMCs is not dependent on the relative prostanoid to cell ratio to 3. In summary, increases in both prostanoid and cell number lowered CM was accounted for by an increase in cell number, medium, cell number was estimated by image analysis using medium in proliferating SMC cultures incubated with CM was accounted for by an increase in cell number, and in fact the relative prostanoid to cell ratio actually decreased (Table 1). Prostanoid synthesis was not required for SMC proliferation (compare medium and CM with and without IM). Furthermore, increased prostanoid synthesis leading to a relative prostanoid to cell ratio of 15 in cultures treated with LPS alone diminished SMC proliferation. This effect was not apparent with 24-hour CM-LPS, probably because the increases in both prostanoid and cell number lowered the relative prostanoid to cell ratio to 3. In summary, enhanced clonal growth with SMCs is not dependent

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell number</th>
<th>6-Keto- PGF₁₀⁻⁰⁻⁻</th>
<th>Prostanoid/ cell</th>
</tr>
</thead>
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<tr>
<td>Medium</td>
<td>100±3 (4)</td>
<td>100±1 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>6-Hour CM</td>
<td>139±6 (4)</td>
<td>121±11 (4)</td>
<td>0.9</td>
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<tr>
<td>12-Hour CM</td>
<td>156±9 (4)</td>
<td>105±3 (4)</td>
<td>0.7</td>
</tr>
<tr>
<td>24-Hour CM</td>
<td>210±5 (4)</td>
<td>189±9 (4)</td>
<td>0.9</td>
</tr>
<tr>
<td>Medium</td>
<td>100±12 (4)</td>
<td>100±9 (4)</td>
<td>1.0</td>
</tr>
<tr>
<td>24-Hour CM</td>
<td>187±11 (4)</td>
<td>95±29 (4)</td>
<td>0.5</td>
</tr>
<tr>
<td>24-Hour CM₄</td>
<td>183±6 (4)</td>
<td>102±4 (4)</td>
<td>0.6</td>
</tr>
<tr>
<td>24-Hour CM₄</td>
<td>194±3 (4)</td>
<td>94±2 (4)</td>
<td>0.5</td>
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<tr>
<td>Medium</td>
<td>100±3 (25)</td>
<td>100±6 (6)</td>
<td>1.0</td>
</tr>
<tr>
<td>+IM</td>
<td>88±3 (26)</td>
<td>23±2 (6)</td>
<td>0.3</td>
</tr>
<tr>
<td>24-Hour CM</td>
<td>166±4 (12)</td>
<td>128±4 (6)</td>
<td>0.8</td>
</tr>
<tr>
<td>+IM</td>
<td>159±7 (12)</td>
<td>28±2 (6)</td>
<td>0.2</td>
</tr>
<tr>
<td>Medium</td>
<td>100±6 (12)</td>
<td>100±22 (12)</td>
<td>1.0</td>
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<tr>
<td>Boiled medium</td>
<td>88±6 (10)</td>
<td>51±6 (10)</td>
<td>0.6</td>
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<tr>
<td>24-Hour, boiled CM</td>
<td>184±10 (10)</td>
<td>136±38 (10)</td>
<td>0.7</td>
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<tr>
<td>Medium</td>
<td>100±9 (5)</td>
<td>100±2 (5)</td>
<td>1.0</td>
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<tr>
<td>+LPS</td>
<td>47±6 (5)</td>
<td>70±43 (5)</td>
<td>15.0</td>
</tr>
<tr>
<td>+LPS+IM</td>
<td>176±15 (6)</td>
<td>9±2 (6)</td>
<td>0.1</td>
</tr>
<tr>
<td>24-Hour CM</td>
<td>117±14 (6)</td>
<td>129±3 (6)</td>
<td>1.1</td>
</tr>
<tr>
<td>24-Hour CM-LPS</td>
<td>293±9 (6)</td>
<td>887±70 (6)</td>
<td>3.1</td>
</tr>
<tr>
<td>+IM</td>
<td>252±9 (6)</td>
<td>82±6 (1)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

6-Keto-PGF₁₀⁻⁻, 6-ketoprostaglandin F₁₀⁻⁻, CM, conditioned medium; IM, indomethacin; LPS, lipopolysaccharide. Rapidly proliferating smooth muscle cells were incubated with medium alone or with CM obtained by preincubation for 6, 12, or 24 hours with either 1 or 2x10⁴ (cells/ml) peripheral blood monocytes (MØs) (adherence) and diluted 1:3 with medium alone. CM₄ was obtained with MØs rewashed with phosphate-buffered saline, and CM₄ was obtained with MØs rewashed with phosphate-buffered saline containing 5 mM EDTA. In some experiments, 5 µg/ml LPS was added during preincubation (24-hour CM-LPS). In other experiments, CM or medium alone was used for 60 minutes. Cells were cloned in medium alone or medium containing 10 µM 1M or 1 µg/ml LPS. The medium was changed on day 5 of the incubation period. After continued incubation for 3 days in fresh medium, cell number was estimated by image analysis using arbitrary units normalized to medium alone (relative cell number), and 6-keto-PGF₁₀⁻⁻ was measured by radioimmunoassay in nanograms per culture and normalized to medium alone (relative 6-keto-PGF₁₀⁻⁻). Normalized data are reported as mean±SEM, and these data were used to calculate a relative prostanoid to cell ratio. Number of incubations is given in parentheses.

Table 1. Soluble Factor in Media Conditioned by Monocytes Stimulates Smooth Muscle Cell Proliferation by a Process Unrelated to Prostanoid Synthesis

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Bar graph showing that prostanoid synthesis in smooth muscle cells is not stimulated by conditioned medium from peripheral blood monocytes (MØs, adherence or Percoll gradient isolations) and alveolar MØs. Confluent cultures of smooth muscle cells were incubated for 24 hours with several concentrations of conditioned medium obtained by 24-hour preincubation with MØs (10⁴ cells/ml). The level of 6-ketoprostaglandin F₁₀⁻⁻ in the medium was measured by radioimmunoassay.

- Confluent SMCs were incubated with CM from peripheral blood MØs isolated either by adherence or Percoll gradient and alveolar MØs. 6-Keto-PGF₁₀⁻⁻ synthesis in SMCs was not affected by CM prepared from MØs preincubated in concentrations as high as 10⁴ MØ per milliliter (Figure 1). These data are not explained by the absence of soluble factors, since the CM used in this study contained soluble factors that stimulated prostanoid synthesis in other cell types. For example, prostanoid synthesis in human neonatal foreskin fibroblasts was increased by CM prepared from alveolar MØs preincubated in concentrations as low as 3 x 10⁴ MØ per milliliter (data not shown). These data confirmed previous studies with soluble MØ factors, which showed that these factors have different effects on SMCs and other cell types.⁵-⁶,⁷,¹⁰,¹¹

- Confluent SMCs were then incubated in cocultures containing either peripheral blood MØs or alveolar MØs. The cocultures all expressed increased amounts of 6-keto-PGF₁₀⁻⁻, and this effect was observed by cell-cell contact with MØs in concentrations as low as 2.5 x 10⁴ MØ per milliliter (Figure 2). Cocultures with peripheral blood MØs isolated by a Percoll gradient had an unexpectedly large stimulatory effect on prostanoid synthesis (Figure 2), but even the CM from these MØs had no effect on prostanoid synthesis in SMCs (Figure 1).

MØs are activated by both adherence to a variety of surfaces and a process in which soluble factors from other cells induce the expression of soluble factors from MØs,⁵,⁶ and it might be possible that MØs are acti-
vated by an interaction with SMCs to express a soluble factor that stimulates PGI\(_2\) synthesis. To test this hypothesis, CM was prepared by preincubation with either SMCs alone or an SMC/MØ coculture. Separate SMC cultures were then incubated with CM prepared in this way, and the data showed that these CM preparations had no effect on 6-keto-PGF\(_{1\alpha}\) synthesis (data not shown). These data confirmed that cell–cell contact was required and that the effect of MØs in cocultures was not the result of the expression of a soluble factor from activated MØs.

The potential effects of soluble SMC factors in activating MØs was tested by another experiment in which cocultures of SMCs and MØs were separated by a Millipore membrane insert. These data show that intact MØs can communicate with SMCs by a soluble factor that is released into the culture medium. The data summarized in Table 2, show that both MØs and an MØ lysate had similar effects, it appears that MØ surface reactive oxygen species by processes that are activated by adherence to biological surfaces. As a consequence, MØs oxidize lipids, and oxygen-centered radicals from oxidized lipids are well known as agents that enhance prostanoid synthesis in SMCs at the cyclooxygenase step in the AA cascade. As a consequence, this study also eliminated the possibility that MØs express a short-lived stimulatory agent that was deactivated during the 24-hour preincubation period before the CM was added to SMCs.

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![Figure 2](image-url)  
**Figure 2.** Bar graph showing that prostanoid synthesis in smooth muscle cells is stimulated in cocultures incubated with peripheral blood monocytes (MØs, adherence or Percoll gradient isolations) and alveolar MØs. The stimulatory effect was greatly enhanced with MØs isolated in a Percoll gradient. Confluent cultures of smooth muscle cells were incubated for 24 hours in cocultures with several concentrations of MØs (10\(^3\) cells/ml). The level of 6-ketoprostaglandin F\(_{1\alpha}\) in the medium was measured by radioimmunoassay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-Keto-PGF(_{1\alpha}) (nmol/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherent MØs</td>
<td></td>
</tr>
<tr>
<td>Medium (no insert)</td>
<td>0.46±0.02 (19)</td>
</tr>
<tr>
<td>MØs (no insert)</td>
<td>1.09±0.07 (13)</td>
</tr>
<tr>
<td>Medium (insert)</td>
<td>0.28±0.02 (15)</td>
</tr>
<tr>
<td>MØs (insert)</td>
<td>0.38±0.01 (14)</td>
</tr>
<tr>
<td>Alveolar MØs</td>
<td></td>
</tr>
<tr>
<td>Medium (no insert)</td>
<td>0.66±0.07 (4)</td>
</tr>
<tr>
<td>MØs (no insert)</td>
<td>1.43±0.07 (6)</td>
</tr>
<tr>
<td>Medium (insert)</td>
<td>0.54±0.06 (4)</td>
</tr>
<tr>
<td>MØs (insert)</td>
<td>0.78±0.03 (6)</td>
</tr>
</tbody>
</table>

Confluent smooth muscle cells were incubated for 24 hours in coculture with peripheral blood monocytes (MØs) (adherence) or alveolar MØs at 5×10\(^3\) cells/ml. Cocultures were compared with and without a Millipore insert. 6-Ketoprostaglandin F\(_{1\alpha}\) (6-keto-PGF\(_{1\alpha}\)) was measured by radioimmunoassay. The number of incubations is given in parentheses.

The stimulatory effect resulting from cell–cell contact (relative content) was not affected by the antioxidant. These data indicate that the generation of lipid peroxides was not responsible for the stimulatory effect of MØs on prostanoid synthesis. MØs and an equal number of lysed MØs were compared for their effect on SMCs in cocultures (Figure 3). The data show that both MØs and lysed MØs enhance PGI\(_2\) synthesis. Furthermore, the stimulatory factor in lysed MØs was not able to pass through a Millipore membrane insert. These data show that intact MØs were not required, and since MØs and an MØ lysate had similar effects, it appears that MØ surface fragments were important in stimulating prostanoid synthesis in SMC cocultures.

**Effect of Cell Contact Between Monocytes and Smooth Muscle Cells on the Arachidonic Acid Cascade in Smooth Muscle Cells**

A number of functional studies were undertaken to examine the effects of cell contact on prostanoid synthesis in SMCs. PGI\(_2\) synthesis (by radioimmunoassay) was enhanced in MØ/SMC cocultures after a 24-hour incubation period even when large amounts of free AA were included in the incubation medium (Figure 4). These data show that the stimulatory effect of cell–cell contact was not overwhelmed by the stimulatory effect of exogenous free AA on PGI\(_2\) synthesis. Similarly, PGI\(_2\) synthesis (by radioimmunoassay) was enhanced in MØ/SMC cocultures after a 24-hour incubation period even when the fatty acid–releasing agent A23187 was included in the incubation medium (Figure 5). These data show that the stimulatory effect of cell–cell contact was not overwhelmed by the stimulatory effect of endogenous AA release on PGI\(_2\) synthesis.

In other experiments, SMCs were prelabeled by the incorporation of \(^{14}\)C\(\)AA into cellular phospholipids, and MØ/labeled-SMC cocultures were then incubated for 24 hours. Total radioactivity measured in the incubation medium was greatly enhanced in cocultures, and this effect was abolished when IM was included in the
TABLE 3. Antioxidant Butylated Hydroxytoluene Does Not Block the Stimulatory Effect of Peripheral Blood Monocytes (Adherence) and Alveolar Monocytes on 6-Ketoprostaglandin $F_{1\alpha}$ Synthesis in Cocultures With Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Treatment (10^5 cells/ml)</th>
<th>No BHT</th>
<th>40 μM BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-Keto-PGF$_{1\alpha}$</td>
<td>nmol/culture</td>
</tr>
<tr>
<td>Adherent MØs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.44±0.03 (10)</td>
<td>100±7</td>
</tr>
<tr>
<td>5.0</td>
<td>0.97±0.01 (3)</td>
<td>220±2</td>
</tr>
<tr>
<td>10.0</td>
<td>0.95±0.06 (9)</td>
<td>216±14</td>
</tr>
<tr>
<td>Alveolar MØs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.58, 0.52</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>1.07, 1.09</td>
<td>196</td>
</tr>
<tr>
<td>5.0</td>
<td>1.34, 1.32</td>
<td>242</td>
</tr>
<tr>
<td>10.0</td>
<td>1.47, 1.40</td>
<td>261</td>
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</table>

Confluent smooth muscle cells were incubated for 24 hours in cocultures with peripheral blood monocytes (MØs) (adherence) or alveolar MØs in the presence or absence of 40 μM butylated hydroxytoluene (BHT). 6-Ketoprostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) was measured by radioimmunoassay and reported as absolute content (nmol/culture) or normalized to medium alone in each experimental series and reported as relative content. The number of incubations are in parentheses; for two incubations, values are reported separately. All other values are mean±SEM.

Incubation medium (Figure 6). Since total radioactivity includes labeled prostanoids, labeled free AA, and other labeled AA metabolites, the IM data strongly suggest but do not prove that only cyclooxygenase metabolites were increased in the medium from cocultures. In other experiments with A23187 and cyclosporine A, IM did not abolish the increase in total radioactivity released to the medium from labeled SMCs. Radioactive metabolites in the medium were then separated by HPLC and shown to consist only of increased amounts of 6-keto-PGF$_{1\alpha}$, PGE$_2$, and three minor fractions that were sensitive to IM and therefore identified as cyclooxygenase products (Figure 7). Similar HPLC tracings (data not shown) were obtained when labeled SMCs were incubated with either LPS or bradykinin. In contrast to these studies, HPLC tracings (data not shown) obtained when labeled SMCs were incubated with either A23187 or cyclosporine A showed increased amounts of 6-keto-PGF$_{1\alpha}$, PGE$_2$, free AA, and many other AA metabolites. Furthermore, labeled free AA and other labeled AA metabolites (data not shown) were greatly increased when IM was included with A23187 or cyclosporine A in the incubation medium. These data showed that MØs only stimulated the formation of cyclooxygenase metabolites in SMCs.

The radioactivity in specific HPLC fractions yielded interesting data (Table 4). Treatment/medium ratios for radioactivity in the 6-keto-PGF$_{1\alpha}$ fraction were increased with both MØ-labeled SMC cocultures and
A23187-labeled SMCs. It is important to note that labeled free AA was not increased in MØ-labeled SMC cocultures (treatment/medium ratio for free AA). Previous studies had shown that labeled free AA was not increased when labeled SMCs were incubated with LPS or bradykinin. There was a striking increase in free AA when labeled SMCs were incubated with A23187 (new data in Table 4) that did not occur when labeled SMCs were cocultured with MØs (compare treatment/medium ratios for free AA). This difference between A23187 and MØs could be explained if MØs stimulated both fatty acid release and cyclooxygenase activity while A23187 stimulated only fatty acid release. However, IM uncouples release from cyclooxygenase activity, and IM did not change the treatment/medium ratio for labeled free AA in MØ cocultures, even though IM further increased the treatment/medium ratio for labeled free AA in cultures treated with the releasing agent A23187. These functional data show that increased prostanooid synthesis in MØ/SMC cocultures was not associated with endogenous fatty acid release.

Discussion

There is little doubt that the progression of the atherosclerotic lesion is a fibroproliferative response to injury that results in the proliferation of aortic SMCs. A number of soluble factors regulate cell proliferation by a final common pathway in which they stimulate cells to express growth-promoting agents such as MØ-derived growth factor, II-1, PDGF, fibroblast growth factor, transforming growth factor-β, and other proliferative agents such as autocrine growth factors, which then induce DNA synthesis. These functional data show that increased prostanoid synthesis in MØ/SMC cocultures was not associated with endogenous fatty acid release.

Studies reported in the present investigation show that MØs express a soluble factor promoting SMC proliferation that is present in media that have been conditioned by preincubation with MØs, is heat labile, and is stimulated by LPS (Table 1). This factor does not stimulate prostanooid synthesis in rapidly dividing cells (Table 1). Furthermore, CM does not contain a soluble factor that induces prostanooid synthesis in confluent SMCs, (Figure 1) even though CM expresses a soluble factor that is highly effective in stimulating prostanooid synthesis in fibroblasts. These studies show that a soluble MØ factor stimulates SMC proliferation without affecting prostanooid metabolism, and as a consequence, the initial proliferative response of SMCs to recruited MØs may be uncoupled from the local regulatory effects of eicosanoid metabolism on SMCs.

Our studies show that MØs themselves have an unexpected effect on eicosanoid metabolism in SMCs in that cell–cell contact between MØs and SMCs results in the stimulation of prostanooid synthesis in SMCs (Table 2 and Figures 2–5). The stimulatory effect of MØs is markedly enhanced when MØs are activated by isolation in a Percoll gradient (Figures 2 and 4). Studies with the antioxidant BHT show that the stimulatory effect of MØs is not merely the result of an oxidative burst in MØs that generates catalytic amounts of hydroperoxy fatty acids (Table 3). These results indicate that increased prostanooid synthesis is an intrinsic property of MØs that is expressed by cell–cell contact with SMCs. Cocultures of bovine SMCs and endothelial cells (ECs) also contain increased amounts of 6-keto-PGF₁α, but this increase is explained by 6-keto-PGF₁α, that is synthesized in ECs, soluble factors such as PDGF from ECs that stimulate prostanooid synthesis in SMCs, and soluble factors from SMCs that stimulate prostanooid
synthesis in ECs. Unlike the SMC/MØ interaction, the SMC/EC interaction involves humoral factors, with cell–cell contact itself having little effect on prostanoid synthesis.

SMCs and MØs accumulate in vascular tissue as the result of the recruitment of peripheral blood MØs and the recruitment and proliferation of SMCs. The increase in these cells within the vasculature increases the potential for cell–cell contact, a process involved in morphoregulation, which is known to regulate cell proliferation in the vessel wall. Cell–cell contact may contribute to the increase in prostanoid levels found in severe atherosclerosis, and increased prostanoid synthesis through cell–cell contact might very well function as a regulatory mechanism for SMC proliferation in established lesions. Atherosclerotic lesions contain unusually high concentrations of 5,8,11-eicosatrienoic acid, which is a well-known characteristic of tissue AA deficiency, and we have previously suggested that uncontrolled atherogenesis may be the result of a relative deficiency in SMC AA that would have as its effect diminished eicosanoid metabolism just when it was needed to regulate SMC proliferation. An SMC AA deficiency would have a particularly significant effect when prostanoid synthesis involved in vascular homeostasis is localized by the functional effect of cell–cell contact between SMCs and MØs.

Prostanoids and hydroxy fatty acids are synthesized in SMCs and other cell lines by the AA cascade. A schematic diagram of the AA cascade (fatty acid release and reincorporation into phospholipids, prostanoid synthesis, and the synthesis of lipoxygenase products and other IM-insensitive metabolites) and sites for the actions of different types of agonists are presented in Figure 8. Early studies suggested that the cascade was controlled by the release of endogenous AA from phospholipids. Several agents, including A23187 and cyclosporine A in MØs and SMCs, and II-1 in mesangial cells, were identified as fatty acid–relasing or type 1 agents (Figure 8). Functional studies with intact cells showed that type 1 agents did not stimulate prostanoid synthesis in the

Figure 7. High-performance liquid chromatography tracings showing that the cell contact factor from peripheral blood monocytes (MØs, adherence) enhances the synthesis of 6-ketoprostaglandin F1α (6-keto-PGF1α), PGE2, and three other cyclooxygenase products (indomethacin sensitive) in smooth muscle cells. Confluent smooth muscle cells were prelabeled with 14C-arachidonic acid and then incubated for 24 hours with fresh medium (upper panel) or medium containing MØs (10^6 cells/ml; lower panel). The five peaks for cyclooxygenase activity were blocked totally by 10 μM indomethacin in medium alone (upper panel) and cocultures with MØs (lower panel), which were concurrent experiments with cells from the same primary culture. Metabolites were extracted, separated by high-performance liquid chromatography, and counted.
Table 4. Cell Contact Factor From Peripheral Blood Monocytes Promotes Prostanoid Synthesis in Smooth Muscle Cells Without Stimulating Free Arachidonic Acid Release

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Metabolite (cpm)</th>
</tr>
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<tbody>
<tr>
<td>M0s</td>
<td>6-Keto-PGF1α</td>
</tr>
<tr>
<td>Medium</td>
<td>45,100</td>
</tr>
<tr>
<td>M0s/medium</td>
<td>17,200</td>
</tr>
<tr>
<td>M0s+1M</td>
<td>2.6</td>
</tr>
<tr>
<td>Medium+1M</td>
<td>1,340</td>
</tr>
<tr>
<td>M0s/medium</td>
<td>0</td>
</tr>
<tr>
<td>A23187</td>
<td>68,700</td>
</tr>
<tr>
<td>Medium</td>
<td>9,370</td>
</tr>
<tr>
<td>A23187/medium</td>
<td>7.3</td>
</tr>
<tr>
<td>A23187+1M</td>
<td>0</td>
</tr>
<tr>
<td>Medium+1M</td>
<td>0</td>
</tr>
<tr>
<td>A23187/medium</td>
<td>...</td>
</tr>
</tbody>
</table>

Confluent smooth muscle cells were prelabeled with $^{14}$C-arachidonic acid (AA) and then incubated with fresh medium alone, with peripheral blood monocytes (M0s, adherence, $10^6$ cells/ml), or $1 \mu$M A23187 in the presence or absence of $10 \mu$M indomethacin (IM). Metabolites were extracted and separated by high-performance liquid chromatography, and the 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) and free AA fractions were counted. Different primary cultures were used with M0s and A23187.

The presence of a large excess of free AA supplied to the cell or released in the cell by other type 1 agents. Type 1 agents stimulated the formation of free AA, and phorbol esters stimulate the formation of free AA from endogenous AA and the formation of cyclooxygenase-independent AA derivatives. Both type 1 and type 2 agents exerted their stimulatory effects on prostanoid synthesis over incubation periods of several hours' duration (References 26 and 30 and D.G. Cornell et al, unpublished observations), but other properties clearly distinguished functional type 1 and type 2 agents in the intact cell.

Some agents, for example, 12-O-tetradecanoylphorbol-13-acetate, exhibit the properties of both type 1 and type 2 agents on the intact cell, and thus, phorbol esters stimulate prostanoid synthesis in the presence of excess free AA, and phorbol esters stimulate the formation of free AA from endogenous AA and the formation of cyclooxygenase-independent AA derivatives. As a consequence, the demonstration of de novo cyclooxygenase synthesis is not sufficient to predict the effects that an agent may have on the intact cell. Functional studies are required to identify the various effects an agent may have on the AA cascade.

The present investigation shows clearly that the functional effects of cell-cell contact between M0s and SMCs on the AA cascade in SMCs are in all ways similar to type 2 agents. Cell contact stimulates 6-keto-PGF$_{1\alpha}$ synthesis in the presence of both large amounts of exogenous AA (Reference 43) and of the powerful type 1 AA-releasing agent A23187 (Reference 5). Cell contact stimulates the release of total radioactivity from prelabeled SMCs, and this effect, as would be expected from agonists that act only at the cyclooxygenase step in the AA cascade, is totally blocked by IM (Reference 6). Cell contact does not increase the amount of labeled free AA (Table 4). Finally, cell contact only stimulates the formation of cyclooxygenase-dependent (IM sensitive) metabolites (Figure 7). It is important to note that cell-cell contact between M0s and SMCs does not generate hydroxy and hydroperoxy derivatives of $^{14}$C- AA from prelabeled cells. M0s express oxygen-centered radicals55,56 that are capable of oxidizing lipids57 in lipoproteins to hydroxy and hydroperoxy derivatives, but these compounds were not found in cocultures of M0s and prelabeled SMCs. Oxygen-centered radicals in oxidized lipoproteins stimulate the formation of large amounts of nonprostanoid oxidation products from prelabeled cells, but unlike preformed lipid peroxides in lipoproteins, M0s have a specific effect on prostanoid synthesis in SMCs rather than a general effect on fatty acid oxidation.

The peripheral blood M0 evidently has important properties that allow it to function effectively in the response-to-injury process that maintains the integrity of the vasculature. M0s express a soluble factor that stimulates SMC proliferation without affecting prostanoid synthesis. Functional studies with intact cells show that later cell-cell contact between recruited M0s...
These functional studies also show that cell-cell contact and proliferating SMCs may generate PGI₂, which is intermediate SMCs and recruited MΟs participates in local increasing as an important part of morpho-
does not generate hydroxy fatty acids, which are capable of enhancing injury. Cell-cell contact is being increasingly recognized as an important part of morpho-
ble of enhancing injury. Cell-cell contact is being increasingly recognized as an important part of morpho-

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Interactions between the monocyte/macrophage and the vascular smooth muscle cell. Stimulation of mitogenesis by a soluble factor and of prostanoid synthesis by cell-cell contact.

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