Response of Atherosclerotic Intimal Smooth Muscle Cells to Epidermal Growth Factor In Vitro

Masako Mitsumata, Shinobu Gamou, Nobuyoshi Shimizu, Yoji Yoshida

Abstract  Increased proliferation of intimal smooth muscle cells (SMCs) plays an important role in the early phase of atherogenesis. To investigate growth mechanisms of these cells, we used intimal SMCs from rabbits fed an atherogenic diet and examined the sequential events that may facilitate induction of intimal SMC proliferation as well as the possible effects of growth-promoting factors secreted by these cells. In serum-free medium, epidermal growth factor (EGF) stimulated [3H]thymidine uptake by quiescent intimal SMCs at a rate six times higher than quiescent medial SMCs. There was no significant difference between the two cell types in terms of the number of specific EGF receptor per cell, the dissociation constant of EGF, and the time course of EGF binding and internalization. Furthermore, in both types of cells, c-fos, c-jun, and c-myc mRNAs were induced after 1, 1, and 4 hours of EGF treatment, respectively, whereas they required 8 hours of contact with EGF to induce proliferation. Growth response of medial SMCs to EGF was greatly enhanced when rabbit serum, deficient in lipoproteins and free of platelet-derived growth factor, was added to the medium. Moreover, EGF induced a twofold to fourfold increase in DNA synthesis in medial SMCs cocultured with intimal SMCs compared with medial SMCs incubated alone. Likewise, DNA synthesis of medial SMCs grown in medium conditioned by intimal SMCs was six times higher than that observed in medium conditioned by medial SMCs. Adding EGF to the medium conditioned by intimal SMCs increased their DNA synthesis even further. These findings suggest that the increased growth potential of intimal SMCs may be regulated by the interaction of EGF and factor(s) secreted by SMCs themselves.

Key Words  • intimal smooth muscle cells  • epidermal growth factor  • receptor binding  • oncogene expression  • growth control

Feeding an atherogenic diet to rabbits for 3 months results in remarkable intimal thickening of the thoracic aorta. Together with extensive fatty deposits and an increase in extracellular matrix, numerous cells identified as smooth muscle cells (SMCs) by a muscle-actin-specific monoclonal antibody appear in the thickened intima. Moreover, incorporation of [3H]thymidine into intimal cell nuclei is relatively high, resulting in a labeling index of 4.71 ± 3.64%, whereas it is negligible in the media. Even though macrophages may be present and some may be labeled, the difference in labeling index between intima and media strongly suggests that intimal SMCs proliferate at a higher rate than medial SMCs. To compare the growth potential of these intimal SMCs with medial SMCs, we developed in vitro cultures of intimal SMCs isolated from explants of thickened intima and compared them with medial SMCs obtained from explants of media. Cellular outgrowth from intimal explants was consistently faster than that from medial explants and, when subcultured, intimal SMCs reached higher cell numbers than comparable medial SMCs.

It is generally accepted that enhanced growth properties of intimal SMCs play an important role in the initiation and progression of human atherosclerosis and that growth factors might accelerate proliferation of vascular SMCs in an autocrine and/or paracrine manner. Thus, we extended our work to examine the growth mechanisms of intimal SMCs in comparison with those of medial SMCs in vitro. Because epidermal growth factor (EGF) is present in platelets and is therefore likely to come in contact with cells in the vascular wall, we examined the effect of EGF on the growth of intimal and medial SMCs and compared it with the effect of fibroblast growth factor (FGF), a known potent angiogenic factor. Our initial studies indicate that intimal SMCs respond to EGF significantly differently than do medial SMCs, whereas both cell types react to FGF to a similar degree. We therefore chose EGF as a stimulant to investigate the growth mechanism of intimal SMCs and examined the sequential events that may facilitate induction of SMC proliferation when stimulated by EGF, as well as the possibility that additional growth-promoting factors may be secreted by intimal SMCs.

Methods

Cell Culture

As described in detail elsewhere, intimal SMCs were isolated from thickened intimas of thoracic aortas of male New Zealand White rabbits that were fed a diet supplemented with 1% cholesterol and 5% lard for 3 to 4 months. Briefly, the endothelial cell layer and adventitia were removed, and the thickened atherosclerotic intima was peeled away from the media with forceps and a razor blade while being viewed under a dissecting microscope. The explants were prepared by cutting the isolated intima into 1- to 2-mm² pieces. For each preparation, at least 15 explants were prepared for histological examination to confirm that they were indeed free of endothelium, media, or adventitia. The media was also isolated from
thoracic aortas of rabbits that were fed standard rabbit chow, and the explants were prepared by methods similar to those used for intimal SMCs. The explants, whether prepared from atherosclerotic intimas or normal medias, were incubated in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). The same medium was used to subculture the outgrowing cells and to establish growth curves; second- or third-passage cultures were used for the experiments. Cells usually reached the stationary growth phase 4 to 5 days after plating. At this time, [3H]thymidine uptake in both intimal and medial SMCs in DMEM with 10% FCS was <10% of the values for day 1. Confluent cells on day 3 or 4 were incubated in DMEM with lipoprotein-deficient rabbit serum (LDS; 1 mg/mL protein) and without platelet-derived growth factor (PDGF) for 24 hours to ascertain quiescence before the cells were used for various experiments.

**Immunocytochemistry**

To confirm the identity of cultured SMCs and the absence of contaminating macrophages, intimal SMCs were stained with monoclonal antibodies for actin filaments and cytoplasmic antigens of tissue-type macrophages by indirect immunofluorescence and a modified avidin–biotinylated peroxidase complex (ABC) method, respectively, by Tsukada et al. Briefly, ABC staining was performed on cells fixed in 4% buffered formaldehyde for 1 minute at room temperature. The antibody, diluted 1:1000, vol/vol, was applied to the cells for 1 hour at room temperature and was followed by serial incubations in 1:500, vol/vol, dilutions of phosphate-buffered saline (PBS) of biotinylated anti-mouse IgG for 1 hour and ABC complex in PBS for 30 minutes. Peroxidase activity was visualized with 3,3′-diaminobenzidine. Muscle-actin-specific (HHF-35), smooth muscle actin-specific (CGA-7), and macrophage-specific (RAM-11) monoclonal antibodies were a gift of Dr. T. Tsukada, Tokyo Medical and Dental University, Tokyo, Japan.

**Growth-Stimulating Factors**

Receptor-grade EGF, extracted from the submaxillary glands of mice, was purchased from Collaborative Research Inc. Basic FGF, extracted from bovine brain, was purchased from Toyobo Co, Ltd. LDS was isolated from the sera of rabbits that were fed standard rabbit chow. After the serum was mixed with an equivalent volume of carboxymethyl–Sephadex C-50 overnight at 4°C to remove any PDGF, the LDS was isolated by ultracentrifugation at a density of 1.24 g/mL and dialyzed against PBS, Hanks' balanced salt solution, and finally DMEM.

Conditioned medium was prepared by plating 8 x 10^5 intimal or medial SMCs into 100-mm culture dishes. When confluent, the cultures were washed extensively with PBS and then incubated for 24 hours in DMEM alone (8 mL per dish). For the next 3 days, this serum-free medium was aseptically collected and centrifuged at 3000 rpm for 15 minutes, and the resulting supernatant was used as intimal SMC–conditioned medium or medial SMC–conditioned medium.

**Cell Proliferation**

Cell proliferation was assayed by either incorporation of [3H]thymidine or the number of cells. To measure [3H]thymidine uptake, 5 x 10^6 cells per well (2.6 x 10^5 cells/cm²) were plated in a 24-well plate. The cells were exposed to 0.2 μCi/mL [3H]thymidine (specific activity, 6.7 Ci/mmol), harvested by trypsin, homogenized, and precipitated with trichloroacetic acid and ether. Radioactivity in the trichloroacetic acid–insoluble fraction was determined with the use of a scintillation counter. An aliquot of homogenate was used to assay the protein concentration by the method of Lowry.

To generate a growth curve, 2 x 10^6 or 1 x 10^6 cells were plated in separate 35-mm dishes, and the cells were counted on days 1, 3, 5, and 7 with use of a hemocytometer. [3H]thymidine uptake was also determined after a 24-hour exposure on selected culture days. To examine the growth reaction to EGF, quiescent cells in 24-well plates were washed twice with PBS and incubated for 48 hours in test medium (1 mL per well) containing [3H]thymidine. To determine the minimum time of contact with EGF necessary to induce proliferation, quiescent cells were stimulated with 25 ng/mL EGF in DMEM with or without LDS (1 mg/mL protein) for selected times, rinsed with PBS, and incubated further in DMEM for a total of 48 hours. [3H]thymidine was present in the medium during the entire incubation period. To test the effect of conditioned media, medial SMCs were incubated for 48 hours in either intimal cell– or medial cell–conditioned medium (1 mL per well) supplemented with either [3H]thymidine or [3H]thymidine and EGF (100 ng/mL). The results of each experiment in this study represent the average of three or four determinations per group. Each experiment was performed at least four times.
Fig 2. Comparison of growth of medial and intimal smooth muscle cells (SMCs) in Dulbecco's modified Eagle medium containing 10% fetal calf serum. A, Bar graph of \(^{[3}H\)thymidine uptake. Medial (○) and intimal (□) SMCs were exposed to \(^{[3}H\)thymidine for 24 hours before harvest. B, Semilog plot of cell numbers per dish. Both medial (——) and intimal (— —) SMCs were plated at two different cell densities in separate dishes. Medium was changed the day after seeding and again on days 3 and 5. Values are mean±SD. *P<.02, **P<.01, #P<.001 vs comparable medial SMCs.

Co-culture
Medial SMCs (5×10^4 per well) were seeded in a 24-well plate and intimal SMCs were placed on a 12-mm-diameter filter with a 0.45-μm pore size (Millicell-HA, Millipore Corp) at densities of 3×10^5 or 4×10^5 cells per filter. Both cell types were cultured separately until confluent in DMEM containing 10% FCS. After an additional 24 hours of incubation in DMEM with LDS (1 mg/mL protein), the cells were rinsed twice with PBS, and a filter with intimal SMCs was placed into each well containing medial SMCs and DMEM with \(^{[3}H\)thymidine (0.2 μCi/mL) with or without added EGF. Thus, the culture medium was shared by both intimal and medial SMCs while they were separated by the filter. For 48 hours of incubation, the intimal SMCs and filters were removed. Medial SMCs were washed thoroughly with PBS, and their \(^{[3}H\)thymidine uptake was measured.

Receptor Binding and Oncogene Induction
Cells were seeded in separate 60- or 100-mm culture dishes at a density of 1.4×10^4 cells/cm^2. The time course for specific receptor binding was analyzed by incubating quiescent cells in 60-mm dishes with 1 ng/mL \(^{125}I\)-labeled EGF in Earle's balanced salt solution containing 0.1% bovine serum albumin at either 4°C or 37°C for selected times. After incubation the cells were washed four times with Earle's balanced salt solution containing 0.1% bovine serum albumin and solubilized in 1 mL 1N NaOH, and the total radioactivity was determined with a gamma counter. Non specific binding was determined in the presence of a 500-fold excess of cold EGF with 1 ng/mL \(^{125}I\)-EGF. A Scatchard plot analysis was performed by incubating the cells for 4 hours at 4°C with 1 ng/mL \(^{125}I\)-EGF with increasing concentrations of unlabeled EGF, resulting in final EGF concentrations that ranged from 0.5 to 100 ng/mL medium.

To examine the mRNA expression of c-fos, c-myc, and c-jun, cells in 100-mm dishes were incubated in DMEM containing 50 ng/mL EGF for 1 to 4 hours. After total cellular RNA was

| Table 1. Effect of Epidermal Growth Factor on \(^{[3}H\)Thymidine Uptake by Medial and Intimal Smooth Muscle Cells in Serum-Free Medium |
|---------------------------------|-----------------|-----------------|-----------------|
| EGF, ng/mL | Medial SMCs (IDPM/μg protein) | Intimal SMCs (IDPM/μg protein) | I/M |
| 0 | 29.9±3.5 | 79.0±7.0* | 2.6 |
| 12.5 | 103.3±6.4 | 710.4±58.0* | 6.9 |
| 25 | 104.0±3.3 | 901.6±113.3* | 8.7 |
| 100 | 141.0±25.2 | 898.2±38.5* | 6.4 |

EGF indicates epidermal growth factor; SMCs indicate smooth muscle cells; and I/M, intimal SMC to medial SMC ratio of \(^{[3}H\)thymidine uptake. Cells were incubated for 48 hours in the test medium containing \(^{[3}H\)thymidine. Values are mean±SD.

*P<.001 vs medial SMCs.

| Table 2. Effect of Basic Fibroblast Growth Factor on \(^{[3}H\)Thymidine Uptake by Medial and Intimal Smooth Muscle Cells in Dulbecco's Modified Eagle Medium With or Without Lipoprotein-Deficient Serum Without Platelet-Derived Growth Factor |
|---------------------------------|-----------------|-----------------|-----------------|
| FGF, ng/mL | Medial SMCs (IDPM/μg protein) | Intimal SMCs (IDPM/μg protein) |
| DMEM | DMEM+LDS | DMEM | DMEM+LDS |
| 0 | 16.0±2.1 | 22.1±1.7 | 24.2±8.1 | 32.6±30.5 |
| 50 | 25.2±1.4 | 62.8±4.3 | 29.4±6.6 | 253.6±44.2 |
| 100 | 34.7±3.9 | 223.8±26.7 | 46.4±5.9 | 240.3±13.1 |
| 200 | 62.9±9.4 | 469.2±16.6 | 66.4±6.3 | 391.8±15.2 |

SMCs indicate smooth muscle cells; FGF, fibroblast growth factor; DMEM, Dulbecco's modified Eagle medium; and LDS, lipoprotein-deficient rabbit serum free of platelet-derived growth factor. Cells were incubated for 48 hours in the test medium containing \(^{[3}H\)thymidine. Value are mean±SD.
Fig 3. Bar graph of minimum contact time with epidermal growth factor (EGF) necessary to induce [3H]thymidine uptake in medial and intimal smooth muscle cells (SMC). Quiescent cells were incubated in Dulbecco's modified Eagle medium (DME) containing EGF (25 ng/mL) and lipoprotein-deficient serum without platelet-derived growth factor (1 mg/mL) (DME+EGF+LDS, a) or EGF alone (DME+EGF, b) for 2, 4, and 8 hours. At indicated times the medium was changed to DME without serum. [3H]thymidine was present for the entire incubation period of 48 hours. Values are mean±SD. *P<.01, **P<.001 vs medial SMC.

Statistical Analysis

All the results of cell proliferation experiments are expressed as mean±SD and were analyzed by Student's t test. Statistical significance was assumed if P<.02.

Results

Immunocytochemical Analyses

The explants were histologically confirmed to consist exclusively of intima as reported previously. We have also shown that during the early period of cell culture, spherical foam cells laden with lipid droplets can be observed among elongated SMCs in the colonies that surround intimal explants. However, these foam cells become detached and gradually decrease in number; the remaining few do not survive trypsinization. Consequently, first-passage cultures consist exclusively of elongated cells. The present study further demonstrates that all elongated first-passage cells react positively with muscle- and smooth muscle actin-specific monoclonal antibodies; no cells have been identified that react with the macrophage-specific monoclonal antibody, although foam cells in primary colonies do react with the macrophage-specific monoclonal antibody (Fig 1).

Growth Curves

To investigate the growth properties of intimal SMCs in further detail, we determined [3H]thymidine uptake and the number of cells under optimal conditions, ie, in DMEM supplemented with 10% FCS. Intimal SMCs showed a 3.6- to 3.8-fold higher uptake of [3H]thymidine on days 1, 2, and 3 and a 3.7-fold (1×10^6 plated cells per dish) and 2.2-fold (2×10^5 cells plated per dish) higher yield in cell number on day 7 than did medial SMCs (Fig 2A and 2B).

Cell Proliferation Stimulated by EGF in Serum-Free Medium and Minimum Contact Time With EGF Needed to Induce Cell Proliferation

In serum-free medium, DNA synthesis of intimal SMCs was also higher than that of medial SMCs. When stimulated by EGF in the range of 0.1 to 200 ng/mL, [3H]thymidine uptake of medial SMCs reached a pla-
teau at a concentration of 50 ng/mL (data not shown). We therefore stimulated cells with EGF in concentration up to 100 ng/mL. Intimal SMCs responded to EGF at a remarkably higher rate than comparable medial SMCs and incorporated 6.4 to 8.7 times as much \[^{3}H\]thymidine as medial SMCs. The intimal SMC to medial SMC ratio of \[^{3}H\]thymidine uptake increased 2.4 times when cells were stimulated by 100 ng/mL EGF (Table 1). In contrast, both cell types reacted to FGF with a similar degree of DNA synthesis, regardless of the presence or absence of LDS (Table 2).

In serum-free medium, the minimum contact time with EGF needed to induce proliferation of intimal SMCs was \(\approx 8\) hours. Similar results were obtained when LDS was added to the aforementioned medium. Although the minimum essential contact time with EGF was identical for intimal and medial SMCs, \[^{3}H\]thymidine incorporation into medial SMCs in serum-free medium in response to EGF was only about one third that of intimal SMCs (Fig 3).

Receptor Binding and Oncogene Induction

The time course of specific EGF binding is shown in Fig 4. EGF binding of both intimal and medial SMCs reached a plateau in 30 to 60 minutes at 4°C, and there was no conspicuous difference between the two cell types in terms of their degree of binding. At 37°C, EGF-binding profiles of intimal SMCs were similar to those of medial SMCs, suggesting that both cell types actively internalize EGF. On Scatchard analysis, both intimal and medial SMCs exhibited high- and low-affinity membrane receptors for \(^{125}\)I-EGF (Fig 5). The number of specific binding sites per cell was 1.2 x 10⁵ for intimal SMCs and 1.3 x 10⁵ for medial SMCs. The dissociation constant was 0.57 nmol/L (high affinity) and 16.7 nmol/L (low affinity) in intimal SMCs and 0.41 nmol/L (high affinity) and 14.0 nmol/L (low affinity) in medial SMCs.

c-fos and c-jun mRNAs were expressed in both intimal and medial SMCs after incubation with EGF for 1 hour. c-myc mRNA was expressed after 4 hours in both cell types as well (Fig 6).

Serum Effect on Cell Growth Stimulated by EGF

Although the growth response of medial SMCs to EGF in serum-free medium was extremely low, addition of LDS to the medium induced a significant response to EGF, resulting in increased \[^{3}H\]thymidine incorporation comparable to that of intimal SMC (Fig 7). These data, together with the observation that intimal SMCs respond to EGF in the absence of LDS, suggest that unlike medial SMCs, intimal SMCs may secrete growth-promoting factors that are similar or identical to the growth factors in LDS. Such factors may cooperate with EGF in inducing cell proliferation in an autocrine manner, even in the absence of serum. To investigate this assumption, we performed further experiments with intimal-medial SMC cocultures and conditioned media from both cell types.

Effect of Coculture and Conditioned Media on Cell Proliferation

EGF in serum-free medium induced a twofold to fourfold higher increase in \[^{3}H\]thymidine incorporation in medial SMCs when cocultured with intimal SMCs
relative to the same cells in monoculture. This still occurred even when the plating density of intimal SMCs was reduced to $3 \times 10^6$ cells per well (Table 3).

When conditioned medium was used to stimulate medial SMCs, [H]thymidine incorporation was six times higher in intimal cell- than in medial cell-conditioned medium: the average uptake of the former was $55.1 \pm 8.8$ disintegrations per minute per microgram protein compared with $8.5 \pm 1.5$ disintegrations per minute per microgram protein for the latter. When EGF (100 ng/mL) was added to these conditioned media, [H]thymidine incorporation in medial SMCs was $163.9 \pm 17.5$ disintegrations per minute per microgram protein in intimal cell-conditioned medium but only $52.1 \pm 3.4$ disintegrations per minute per microgram protein in medial cell-conditioned medium. That is, intimal cell-conditioned medium and EGF alone increased [H]thymidine uptake in medial SMCs about sixfold, whereas their combined effect was roughly 20-fold (Fig 8).

Discussion

The features that most distinguish cultured intimal SMCs from medial SMCs in our in vitro studies are morphology\(^7\) and growth properties. Proliferation of intimal SMCs was higher than that of medial SMCs in any medium containing either no serum, FCS, normal rabbit serum, hyperlipidemic rabbit serum, or LDS.\(^7,17\) To investigate early atherogenesis, we used medial SMCs from rabbits that were fed normal chow. However, in a previous study we compared medial and intimal SMCs from thoracic aortas of rabbits fed a high-fat diet and we obtained similar results.\(^7\) Similar findings have also been reported by other investigators who used intimal SMCs isolated from thickened intimas formed as a result of endothelial denudation.\(^18\) These results indicate that, even after being isolated and subcultured in vitro, intimal SMCs preserve their in vivo characteristics of proliferation.

**Table 3. Effect of Intimal Smooth Muscle Cells on [\(H\)]Thymidine Uptake by Medial Smooth Muscle Cells**

<table>
<thead>
<tr>
<th>[(H)]Thymidine Uptake, Disintegrations per Minute per Microgram Protein</th>
<th>With Intimal SMCs, Cells per Well</th>
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<tr>
<td>EGF, ng/mL</td>
<td>Without Intimal SMCs</td>
</tr>
<tr>
<td>0</td>
<td>13.4±1.3</td>
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<tr>
<td>12.5</td>
<td>62.2±8.6</td>
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<tr>
<td>100</td>
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Medial smooth muscle cells (SMCs) were incubated either alone or in coculture with intimal SMCs in Dulbecco's modified Eagle medium with [\(H\)]thymidine with or without epidermal growth factor (EGF). After 48 hours, [\(H\)]thymidine uptake by medial SMCs was determined. Values are mean±SD.

\(^{*}\)P<.01, \(^{t}\)P<.001 vs absence of intimal SMCs.

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Fig. 7. Bar graphs showing effect of increasing concentrations of epidermal growth factor (EGF) on intimal (right) and medial (left) smooth muscle cells (SMC) in the presence of lipoprotein-deficient serum without platelet-derived growth factor (LDS). Quiescent SMC were incubated in Dulbecco's modified Eagle medium containing EGF, LDS, and [\(H\)]thymidine for 48 hours. Values are mean±SD.

Fig. 8. Bar graph showing effect of conditioned medium and epidermal growth factor (EGF) on [\(H\)]thymidine uptake of medial smooth muscle cells (SMCs). Conditioned medium was prepared from either medial (M-CM; □) or intimal (I-CM; ●) SMCs. Quiescent medial SMCs were then incubated in either M-CM or I-CM with or without EGF. Values are mean±SD.

\(^{*}\)P<.001 vs M-CM.
EGF stimulates replication of quiescent arterial medial SMCs\(^{19-23}\) similar to that of other mesenchymal cells.\(^{24,25}\) It is generally assumed that the addition of serum is necessary for EGF to stimulate proliferation of SMCs.\(^{22}\) In fact, medial SMCs in this study responded well to EGF when incubated in DMEM with LDS. However, in serum-free medium, the proliferative response to EGF (100 ng/mL) was only \(\approx 11\%\) of that obtained with serum. EGF-induced S phase entry in intimal SMCs, even in serum-free medium, that was comparable to that obtained in medium with serum added.

Because the minimum contact time with EGF necessary to induce cell proliferation was about 8 hours, we examined the cellular events that were evoked earlier than 8 hours. In agreement with other reports,\(^{19,26,27}\) both intimal and medial SMCs have receptors for EGF. Resink et al\(^ {26}\) have shown that SMCs obtained from thoracic aortas of spontaneously hypertensive rats proliferate at a higher rate than do cells from Wistar-Kyoto rats and show a higher degree of specific receptor binding of \(\beta^1\)-EGF. In our study, there was no obvious difference in the receptor number and affinity of EGF in the time course of receptor binding for EGF, and both cell types exhibited high- and low-affinity surface membrane receptors. Furthermore, there was no significant difference between the two types of SMCs in either the number of specific binding sites per cell or the dissociation constant. In other words, the difference in the growth pattern of intimal and medial SMCs in response to EGF cannot be attributed to differences in receptor binding.

It is assumed that growth factors, such as PDGF, and tumor promoters, such as phorbol 12-myristate 13-acetate, transiently increase DNA binding proteins that specifically recognize the promoter or enhancer region of the gene and that this increase in DNA binding proteins acts as a signal for cell proliferation.\(^ {28,29}\) Similar to PDGF, EGF accelerates induction of c-myc and c-fos mRNA in fibroblasts,\(^ {30,31}\) 3T3 cells,\(^ {24,31}\) and hepatocytes.\(^ {32}\) Only Burden et al\(^ {33}\) and Bukac et al\(^ {34}\) have demonstrated EGF-stimulated expression of c-myc and c-fos mRNA in vascular SMCs. In this study, we investigated the expression of c-fos, c-jun, and c-myc mRNA in response to EGF in medial and intimal SMCs and found that both cell types express the three proto-oncogenes in almost the same manner. Moreover, although both types of SMCs express proto-oncogenes within 4 hours of EGF stimulation, it is not until after 8 hours of contact with EGF that DNA synthesis is induced. These results suggest that mRNA expression of c-fos, c-jun, and c-myc may not be directly coupled to the induction of enhanced cell proliferation of intimal SMCs.

It has been shown that vascular SMCs produce and secrete growth factors, such as PDGF\(^ {35-37}\) and insulin-like growth factor,\(^ {38}\) and thrombospondin,\(^ {20}\) which may synergistically accelerate proliferation with EGF in an autocrine and/or paracrine manner. On the other hand, SMCs also secrete growth inhibitors, such as heparan sulfate moieties.\(^ {39,41}\) In this study, intimal SMCs but not medial SMCs secreted a substance into the medium that promoted proliferation of medial SMCs and cooperated with EGF to further enhance proliferation of intimal SMCs. In contrast to the effect of the medium conditioned with intimal SMCs, coculture with intimal SMCs did not promote DNA synthesis in medial SMCs unless EGF was also present. This lack of effect is probably due to an insufficient amount of growth-promoting factors secreted by the limited number of intimal SMCs on the small area of the filter. Our results suggest that growth-promoting substances secreted by intimal SMCs may be factors, other than PDGF and lipoproteins, that are normally present in rabbit serum.

It is also possible that substances secreted by intimal SMCs may act as antagonists to growth inhibitors, such as heparin, which prevents the transition of SMCs from the G\(_0/G_1\) to the S phase. Although EGF was shown to overcome heparin inhibition, this was ineffective when SMCs were pretreated with heparin for 48 hours before the addition of EGF.\(^ {22}\) Thus, it appears that quiescent medial SMCs, whose growth is suppressed in an autocrine manner by heparin-like substances, are enabled by EGF to proceed from the G\(_0\) to the G\(_1\) phase and to express mRNA proto-oncogenes but cannot proceed to the S phase in the absence of serum. Intimal SMCs, on the other hand, proceed to the S phase under EGF stimulation even in the absence of serum; this difference is conceivably due to the secretion of some as-yet-unknown substance(s) that can inactivate the growth inhibitors.

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References


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