Regulation of Insulin-like Growth Factor I Messenger RNA Levels in Vascular Smooth Muscle Cells

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We have previously demonstrated specific insulin-like growth factor I (IGF I) messenger RNA (mRNA) transcripts in cultured rat aortic smooth muscle cells (RASM). To define the role of IGF I in the autocrine growth program of vascular smooth muscle cells, we quantitated IGF I mRNA levels in proliferating and quiescent (serum-deprived for 48 hours) RASM. IGF I mRNA levels were markedly decreased in quiescent cells, and this effect was reversible on reexposure to serum. Since platelet-derived growth factor (PDGF) acts synergistically with IGF I to stimulate vascular smooth muscle cell growth, we exposed quiescent RASM to PDGF AB or BB and quantitated IGF I transcript levels. Both PDGF dimers caused a marked, rapid increase in IGF I message levels. To determine whether induction of IGF I mRNA levels correlated with secretion of IGF I, we measured immunoreactive IGF I in RASM conditioned medium after separation of IGF I binding proteins by gel filtration chromatography. PDGF caused a significant increase in IGF I release at 24 hours. These findings indicate that IGF I mRNA levels in vitro are regulated by serum and by growth factors such as PDGF. Serum deprivation reversibly decreases IGF I transcript levels, and exposure of quiescent cells to PDGF increases IGF I mRNA levels and IGF I release. Regulation of IGF I expression by competence growth factors such as PDGF may play an important role in the control of vascular smooth muscle cell growth. (Hypertension 1991;18:742–747)

Proliferation of vascular smooth muscle cells (VSMC) is a key feature of several pathogenic processes including atherosclerosis, the vessel wall response to hypertension, and restenosis after coronary angioplasty. Autocrine/paracrine mechanisms that may be important in growth responses of the vessel wall include stimulation of cellular division/hypertrophy by peptide growth factors that can be released from platelets at sites of vessel injury as well as produced by VSMC and endothelial cells. Evidence supporting their role has emerged from data demonstrating the presence of messenger RNA (mRNA) for platelet-derived growth factor (PDGF) and for its receptor in intimal cells in human atherosclerotic plaque and from the observation that luminal endothelial cells and intimal smooth muscle cells in healing vascular grafts express PDGF A-chain mRNA. Furthermore, balloon injury of rat aorta induces insulin-like growth factor I (IGF I) gene expression. In vitro data indicate that these two growth factors are potent co-mitogens acting synergistically to promote VSMC growth. Furthermore, Clemmons and co-workers have shown an increase in IGF I-like activity in conditioned medium from fibroblasts and VSMC exposed to PDGF. Our laboratory has recently demonstrated the presence of specific IGF I mRNA transcripts in cultured rat aortic smooth muscle (RASM) and bovine aortic endothelial cells. In the present report, we characterize regulation of IGF I mRNA levels and secretion in RASM. Our findings indicate that serum deprivation of RASM for 48 hours causes a marked reduction in IGF I mRNA levels and that exposure of quiescent RASM to PDGF AB or BB causes a rapid and sustained increase in IGF I mRNA levels. This is accompanied by an increase in IGF I release into RASM conditioned medium. Our data provide strong evidence that IGF I mRNA levels in VSMC are regulated by serum and by PDGF. An autocrine “loop” in which PDGF mediates induction of an endogenous progression factor such as IGF I may

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be important in disease processes such as hypertension and atherosclerosis in which VSMC proliferation is a central feature.

**Methods**

**Cell Culture**

VSMC were isolated from rat thoracic aorta as described previously. Cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg streptomycin. They were passaged one to two times a week by harvesting with trypsin-verse and seeding in 80-cm² flasks. For experiments, cells between passages 5 and 18 were seeded in 100-mm dishes (2×10⁶ cells/cm²), fed every other day, and used at 90% confluence (3–6 days). Quescence was approached by washing 80–90% confluent cells with serum-free medium (SFM) twice followed by incubation in SFM for 48 hours. This has been shown in our laboratory to decrease [³H]thymidine incorporation by more than 75%. To determine the effects of serum on IGF I mRNA levels, quiescent cells were exposed to SFM or to DMEM containing 10% calf serum for 24 hours. For some experiments, quiescent cells were exposed to fresh SFM in the absence or presence of PDGF AB (10 ng/ml) or PDGF BB (10 ng/ml), and cell-conditioned medium and cells were harvested at various times for radioimmunoassay and RNA extraction, respectively.

**Radioimmunoassay**

Specific IGF I immunoreactivity of cell-conditioned medium was determined as previously described by our laboratory. In brief, medium was dialyzed, lyophilized, resuspended in 1 M acetic acid/0.025 M NaCl, incubated at room temperature for 1 hour, and then chromatographed using Biogel P30 polyacrylamide columns (Bio-Rad Labs, Richmond, Calif.). This permits separation of IGF I from its binding proteins. IGF I fractions elute in a second peak and were assayed using a polyclonal anti-IGF I antibody antiserum kindly provided by Dr. L. Underwood and J.J. Van Wyk through the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. A double antibody technique was used to precipitate bound counts using goat antirabbit antiserum and normal rabbit serum as a carrier. Standard curves were generated using human recombinant IGF I (Amgen). All experiments were performed at least three times. The recovery loss of IGF I using Biogel columns was consistently less than 14%.

**Insulin-like Growth Factor I Gene Expression**

Total RNA was extracted from cultured cells using the guanidium isothiocyanate/cesium chloride method. RNA was quantitated by spectrophotometry and only RNA with 260/280 o.d. ratios of 1.8–2.0 was used. Purity of RNA was additionally verified by agarose gel electrophoresis and ethidium bromide staining. For Northern blot analysis, RNA was enriched in polyadenylated (A+) RNA by two passages on an oligothymidylic acid cellulose column, and 10–20 μg of total or A+ RNA was denatured with DMSO/glyoxal and was size-fractionated by agarose gel electrophoresis before transfer to a nylon membrane (Genescreen Plus, New England Nuclear, Boston, Mass.). Membranes were prehybridized at 65°C for 3–5 hours then hybridized for 24 hours in a solution containing 2.5× Denhardt's ([1× Denhardt's: 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin], 1 M NaCl, 2 mM EDTA, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 100 μg/ml denatured herring DNA, and 10⁶ cpm/ml of a denatured IGF I complementary DNA (cDNA) probe. The IGF I cDNA was a rat 521 base pair fragment including the entire coding sequence for IGF I and was kindly provided by Dr. G.I. Bell, Howard Hughes Medical Institute, University of Chicago. The cDNA was labeled by random priming using [³²P]dCTP and Klenow enzyme. After hybridization, filters were washed with 2× standard saline citrate (SSC) ([1× SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7] at room temperature, 2× SSC + 1% SDS at 55°C and finally in 0.1× SSC + 0.1% SDS at 55°C before autoradiography. The molecular sizes of detected RNA species were determined from the migratory pattern of ribosomal RNA. Additionally, to control for differences in gel loading, filters were hybridized with a 28S ribosomal probe. Solution hybridization/RNase protection assays were performed as described by Melton et al. In brief, 40 μg total RNA was hybridized to a [³²P]UTP-labeled antisense riboprobe generated by T7 polymerase transcribe of a linearized plasmid containing the rat exon 3 and adjacent intron sequences. The plasmid was kindly provided by Dr. P. Rotwein, Washington University School of Medicine. Hybridization was performed overnight in a solution containing 80% deionized formamide, 50 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA, and 0.5–1× 10⁶ cpm riboprobe. After RNase digestion using 40 μg/ml ribonuclease A and 50 units/ml ribonuclease T1, samples were proteinase K treated, phenol extracted, ethanol precipitated, and analyzed on a denaturing polyacrylamide urea (sequencing) gel. The full-length probe is 956 bp, and the protected fragment is 182 bp in length. Autoradiographic bands were quantitated by twodimensional laser densitometry. To verify the integrity of the RNA and to ensure that equal amounts were loaded, 10-μg aliquots of RNA were subjected to agarose gel electrophoresis and ethidium-bromide staining before hybridizations. All experiments were performed at least three times.

**Results**

As previously demonstrated by our laboratory, Northern hybridization analysis of A+ RNA from RASM yields three primary IGF I mRNA transcripts sized 7.5 kb, 1.7 kb, and 0.9–1.2 kb (Figure 1). When
FIGURE 1. Northern analysis of insulin-like growth factor I (IGF I) gene expression in rat aortic smooth muscle cells. RNA was prepared from cells grown to 80–90% confluence in Dulbecco's modified Eagles medium with 10% calf serum (CS) or quiesced by exposure to serum-free medium (SFM) for 48 hours. Polyadenylated RNA (A+) (15 µg) was hybridized to a phosphorus-32-labeled IGF 1 complementary DNA (cDNA) probe. Upper panel shows three primary IGF I messenger RNA transcripts and their sizes as estimated from the positions of 28S and 18S ribosomal RNA on the ethidium-bromide stained gel, shown in kilobases. There was a decrease in transcript levels in quiescent cells. Lower panel shows an autoradiogram of the blot hybridized to a 28S ribosomal cDNA probe as a control for loading.

RASM grown in DMEM with 10% calf serum were serum-deprived for 48 hours, there was an inhibition of IGF I mRNA levels when compared with cells maintained in 10% calf serum. This is demonstrated by Northern analysis in Figure 1. To quantitate the decrease in IGF I mRNA levels, we used a highly sensitive solution hybridization/RNase protection assay. We have previously used this technique to demonstrate IGF I gene expression in cultured VSMC.11 After hybridization of RASM RNA to the antisense IGF I riboprobe and RNase digestion, a protected 182 bp fragment is visible as shown in Figure 2. Quantitation of protected bands by two-dimensional laser densitometry demonstrated a 68±9% (mean±SEM, n=3, p<0.01) decrease in IGF I mRNA levels in RASM that were serum-deprived for 48 hours as compared with cells maintained in 10% calf serum. This effect was reversible since exposure of quiescent RASM to DMEM containing 10% calf serum for 24 hours caused an 88% increase in IGF I mRNA levels (mean of determinations from two experiments).

To determine the effect of PDGF on IGF I mRNA levels in quiescent cells, VSMC were grown to 90% confluence in 10% calf serum, serum-deprived for 48 hours, and then exposed to fresh SFM for an additional 24 hours in the presence or absence of PDGF AB (10 ng/ml) or PDGF BB (10 ng/ml). Solution hybridization/RNase protection analysis was performed, and a representative experiment is shown in Figure 2. Quantitation of IGF I mRNA levels by two-dimensional laser densitometry indicated a rapid and sustained increase in IGF I mRNA levels in RASM exposed to PDGF (Figure 3). The absolute increase in transcript levels peaked at 6 hours and
was sustained for at least 24 hours. IGF I mRNA levels in quiescent cells exposed to PDGF BB and PDGF AB for 24 hours increased by 109±37% (p<0.05) and by 93±30% (p<0.05), respectively, when compared with cells exposed to SFM alone. The PDGF-induced increase in IGF I mRNA levels above those in cells exposed to SFM was of borderline statistical significance at 6 hours but attained statistical significance (p<0.05) at 12 hours. The addition of fresh SFM to quiescent cells caused a lesser but significant increase in IGF I mRNA levels (p<0.05 at all time-points), but these decreased toward baseline at 24 hours.

To determine whether the PDGF-stimulated increase in IGF I mRNA in quiescent cells correlated with an increase in IGF I protein, quiescent RASM were exposed to fresh SFM for 24 hours in the presence or absence of PDGF AB or BB. IGF I in the cell-conditioned medium was measured using a highly specific radioimmunoassay, performed after separation of IGF I from its binding proteins by gel filtration chromatography, as we have previously described. As shown in Figure 4, there was a substantial basal release of IGF I in SFM, and this was increased in the presence of PDGF. This increase averaged 83% and 53% at 24 hours for PDGF BB and AB, respectively (p<0.05).

Discussion

IGF I is a progression growth factor that acts primarily on the G1 phase of the cell cycle to promote entry into the S phase. As such, its effects are synergistic with competence factors such as PDGF and fibroblast growth factor (FGF) that act primarily by stimulating entry into G1. Initial studies demonstrating release of IGF I-like immunoactivity from VSMC have been substantiated and extended by recent data from our laboratory demonstrating specific IGF I gene expression in cultured VSMC. The potential importance of endogenous production of IGF I has been demonstrated by studies showing that growth hormone– and PDGF-stimulated growth of fibroblasts and VSMC may be inhibited by anti-IGF I monoclonal antibody.

As an initial approach to define the regulation of VSMC IGF I mRNA levels, we quantitated message levels in cells proliferating in 10% calf serum and in cells rendered quiescent by serum deprivation for 48 hours. There was a dramatic decrease in IGF I mRNA levels in these cells. Reexposure of quiescent cells to 10% calf serum for 24 hours reversed this effect. It is of note that exposure of quiescent cells to fresh SFM caused an initial transient increase in IGF I mRNA levels. Analogous to this is our finding that during the initial phase of serum deprivation, there was a transient increase in IGF I mRNA levels (i.e., levels measured 6 hours after the initiation of serum deprivation were increased when compared with starting levels, data not shown). However, after 48 hours of serum deprivation there was a dramatic 68±9% decrease in IGF I mRNA levels when compared with levels in cells in the presence of 10% serum.

There are both similarities and differences between our findings and recently published data regarding IGF I mRNA levels in cultured fibroblasts and VSMC. Lowe et al have shown that exposure of fibroblasts deprived of serum for 24 hours to fresh SFM increases IGF I gene expression. This is similar to our effect in cells deprived of serum for 48 hours; however, these authors also reported that medium containing serum decreased IGF I mRNA levels in those cells at 18 hours, whereas in our quiescent VSMC the addition of 10% calf serum markedly increased IGF I mRNA levels. This difference may well be explained by the fact that in the study of Lowe et al, the fibroblasts were less quiescent (24-hour serum deprivation) and that IGF I mRNA levels in actively proliferating cells (before quiescence) were not reported. A recent study from Bornfeldt et al reported an increase in IGF I mRNA levels (as determined by solution hybridization) in VSMC exposed to sequential 24-hour periods of serum deprivation and a decrease in IGF I mRNA levels in cells exposed to serum. This difference may again be explained by the way in which these authors deprived their cells of serum. Indeed, we show in Figure 3 that the exposure of quiescent cells to fresh SFM transiently increases IGF I mRNA levels.

In an effort to provide further insights into IGF I gene regulation, we studied the effects of PDGF on IGF I gene expression. Prior studies from Clemmons and coworkers demonstrated increased IGF I immunoreactivity in conditioned medium from VSMC and fibroblasts exposed to PDGF. Additionally, recent data have shown that PDGF stimu-
lates release of IGF I immunoreactivity from rat liver fat-storing cells and that this immunoreactivity may in part be accounted for by an increase in IGF I binding protein activity.25 Our findings demonstrate that both PDGF AB and BB cause a sustained increase in IGF I mRNA levels in quiescent cells. These data differ from those reported by Bornfeldt et al24 showing a small decrease in IGF I mRNA levels in VSMC exposed to PDGF BB for 24 hours. The reason for this difference is not clear, but it is of note that in the latter study, VSMC were serum-deprived for only 24 hours before exposure to PDGF, and therefore, it is possible that steady-state basal IGF I mRNA levels were not achieved. To demonstrate the functional relevance of the data, we measured secreted IGF I protein levels by radioimmunoassay and found a significant increase 24 hours after PDGF exposure. In a recent abstract, Giannella-Neto et al26 have likewise reported an increase in IGF I mRNA levels in VSMC exposed to PDGF isoforms AA, BB, or AB.

Our findings that IGF I mRNA levels in VSMC are regulated by serum and by PDGF may have particular relevance to a variety of important disease processes in which VSMC proliferation is a central feature, notably atherosclerosis, hypertension, and restenosis after angioplasty. Although endothelial denudation is not a prerequisite for the development of atherosclerosis, one of the early lesions in the atherosclerotic process is migration of monocytes into the subendothelial space.27 Monocytes are transformed into activated macrophages that may release growth factors such as PDGF. One may speculate that this plays a key role in the VSMC proliferative response through stimulation of IGF I gene expression. Certain forms of hypertension such as abdominal coarctation hypertension in the rat are thought to represent a form of acute vessel wall injury and are accompanied by endothelial cell and VSMC hyperplasia.28 Our laboratory has recently demonstrated a marked increase in IGF I gene expression in aortic endothelial cells and VSMC in this model,29 consistent with a role for IGF I in the hypertensive remodeling of the vessel wall. Recent data from Cercek et al10 have demonstrated a marked increase in IGF I mRNA levels in rat aorta after balloon injury. Since the response to vessel wall deendothelialization includes platelet aggregation, one may again speculate on the potential importance of PDGF release from activated platelets and subsequent induction of IGF I gene expression. Alternatively, PDGF could be produced by VSMC and trigger synthesis of its own progression factor through stimulation of IGF I expression.

In summary, our data indicate that IGF I mRNA levels are related to the growth state of the VSMC in vitro. Serum deprivation for 48 hours markedly decreases IGF I mRNA levels, and this effect is reversible. One of the important mitogens contained in serum, PDGF, markedly increases IGF I transcript levels and IGF I release in quiescent VSMC. We are at present investigating whether the regulation of IGF I mRNA by serum and PDGF is at the transcriptional or posttranscriptional level. These findings are consistent with a central role for IGF I in the endogenous growth program of the blood vessel wall and may be key to understanding mechanisms of VSMC proliferation in disease processes such as hypertension, atherosclerosis, and restenosis after angioplasty.

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