Insulin-like growth factor I (IGF I), a potent growth factor in vitro, is present in blood and in multiple tissues and is a major mediator of the effects of growth hormone on postnatal growth. IGF I is internalized and retained largely intact in cultured vascular endothelial cells. Neovasculature transiently expresses IGF I immunoreactivity, but it is not known whether this represents internalization of the circulating growth factor or vascular cell synthesis of IGF I. As an initial approach to defining the role of endogenous production of IGF I in the growth program of the vessel wall, Northern hybridizations were performed with RNA from cultured rat aortic smooth muscle cells and bovine aortic endothelial cells. Rat aortic smooth muscle cells expressed three primary IGF I messenger RNA transcripts sized 8.2, 1.7, and 0.9-1.2 kb. Bovine aortic endothelial cells expressed one major and one minor IGF I transcript of 2.1 and 1.6 kb, respectively. IGF I gene expression in smooth muscle cells was also demonstrated by ribonuclease protection assays using a rat exon 3 riboprobe. Both endothelial and vascular smooth muscle cells secreted IGF I, as detected by radioimmunoassay of conditioned medium after separation of IGF I from its binding proteins by gel filtration chromatography. Because IGF I stimulates growth of vascular cells, characterization of IGF I gene expression in blood vessels may be key to understanding developmental as well as abnormal growth in the cardiovascular system. (Hypertension 1991;17:693-699)
least three different 5' untranslated regions (class A,B,C) and one of two different 3' domains, we have used a rat IGF I complementary DNA (cDNA) covering the common IGF I coding sequence. Hybridization studies demonstrated the presence of three primary IGF I mRNA transcripts in cultured rat aortic smooth muscle cells (RASM) and two primary IGF I mRNA transcripts in cultured bovine aortic endothelial cells (BAEC). Radioimmunoassay of conditioned medium from BAEC and RASM after separation of binding proteins by gel filtration chromatography demonstrated specific IGF I immunoactivity. These findings demonstrate specific IGF I gene expression in vascular endothelial and smooth muscle cells and provide inferential evidence that vascular cells, through the production of IGF I, may have a broader potential than previously defined for exerting paracrine or autocrine growth effects both in the blood vessel wall as well as on nonvascular parenchymal cells.

Methods

Cell Culture

VSMC were isolated from rat thoracic aorta as described previously. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum, 2 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin. They were passaged one to two times a week by harvesting with trypsin-versene and seeding in 80-cm² flasks. Cells were used at passage levels (3-6 days). For experiments, cells between passages 5 and 18 were seeded in 100-mm dishes (2×10⁵ cells/cm²), fed every other day, and used at 90-100% confluence (3-6 days).

Endothelial cells were prepared from bovine aorta as described by Gimbrone. Primary cultures were grown in DMEM supplemented with 10% fetal calf serum, glutamine and antibiotics and were passaged in 80-cm² flasks. Cells were used at passage levels 3-18 after replicate plating into 100-mm dishes. Purity of cultures was determined by acetylated low density lipoprotein (LDL) uptake and only cultures free of any contaminating cells were used.

Radioimmunoassay

Ninety to 100% confluent cells were washed three times in serum-free medium and incubated in serum-free medium for 24 hours. Conditioned medium was dialyzed, lyophilized, and resuspended in 1 M acetic acid/0.025 M NaCl and was chromatographed using Biogel P30 polyacrylamide (Bio-Rad, Richmond, Calif.) columns. Fractions were assayed for IGF I immunoreactivity using a polyclonal anti-IGF I antibody 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 anti-rabbit antiserum and normal rabbit serum as a carrier. Standard curves were generated using human recombinant IGF I (Amgen). IGF I immunoreactivity was expressed as a function of cell protein, which was determined by the method of Bradford.

Binding Protein Assay

IGF I binding activity of conditioned medium after Biogel P30 chromatography was determined using a charcoal assay. Briefly, 100-μl aliquots from each column fraction were incubated with 125I-IGF I (~20,000 cpm) in an assay buffer containing 0.25% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4°C overnight, and unbound counts were precipitated using 0.25% (wt/vol) activated charcoal. Counts in the supernatant (bound by binding proteins) and in the pellet (bound by charcoal) were quantitated by automated gamma counting. Specific IGF I binding activity was expressed as a percentage of total binding activity. Nonspecific binding was determined by incubating 125I-IGF I with activated charcoal in the absence of sample.

Insulin-Like Growth Factor I Gene Expression

Total RNA was extracted from cultured cells with the guanidium isothiocyanate/cesium chloride method and enriched in polyadenylated RNA by two passages on an oligothymidilic acid cellulose column. Ten to 20 μg of total RNA, polyadenylated RNA (A+), or A- RNA [RNA left after two passages on oligo(dT) cellulose] were denatured with dimethyl sulfoxide/glucosyl and size-fractionated by agarose gel electrophoresis before transfer to a nylon membrane. 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% BSA), 1 M NaCl, 2 mM EDTA, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), 100 μg/ml of denatured herring DNA, and 10⁶ cpm/ml of a denatured IGF I cDNA probe. The IGF I cDNA was a rat 521 base pair fragment, which included 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× 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. Only RNA with an optical density 260/280 ratio of 1.8-2.0 and with no signs of degradation on ethidium-bromide staining was used for experiments. Purity of RNA was additionally verified by hybridization to a β-actin probe, which gave a clear band.

To further demonstrate IGF I gene expression in VSMC, solution hybridization/ribonuclease (RNase) protection assays were performed as described by Melton et al. Forty micrograms total RNA were hybridized to a [32P]UTP-labeled antisense riboprobe.
FIGURE 1. Line graphs showing immunoreactive insulin-like growth factor I (IGF I) and IGF I binding proteins in rat aortic smooth muscle cells (RASM) conditioned medium. Panel A: Radioimmunoassay (RIA) of RASM-conditioned medium. Confluent cells were washed in serum-free medium and exposed to serum-free medium for 24 hours. Conditioned medium was dialyzed, acidified, and chromatographed on polyacrylamide columns before RIA. Two elution peaks were present and, as shown in panel B, the first peak corresponded to binding proteins and the second peak to immunoreactive IGF I. Panel B: Aliquots from the chromatography fractions were assayed for presence of IGF I binding protein activity by a charcoal assay (broken line). Solid line shows elution profile of human recombinant $^{125}$I-IGF I.

Results

Both RASM and BAEC released IGF I as determined by radioimmunoassay of conditioned medium. Figure 1A demonstrates the elution profile of RASM-conditioned medium after dialysis, acidification, gel filtration chromatography, and radioimmunoassay. Two peaks of activity were present, and BAEC-conditioned medium yielded a similar profile.

Serum-free medium and the third wash of serum-free medium before incubation of the cells in serum-free medium for 24 hours contained no detectable IGF I. Plasma and conditioned medium from various cell types including endothelial cells have been found to contain significant amounts of IGF I binding proteins that are known to cross-react in the radioimmunoassay by displacing tracer $^{125}$I-IGF I from the anti-IGF I antibody. We thus measured the binding protein activity of the chromatographed fractions using a charcoal assay. Only one peak of binding protein activity was present in both endothelial cell- and VSMC-conditioned medium, and this peak corresponded closely to the first peak on the IGF I immunoreactivity profile (Figure 1B). Furthermore, the elution profile of human recombinant $^{125}$I-IGF I after acid chromatography corresponded to the second peak of IGF I immunoreactivity. Serial dilutions of the pooled fractions from the second peak of IGF I immunoreactivity from BAEC- and RASM-conditioned medium displaced iodinated tracer IGF I from antibody in a manner parallel to standard (not shown). These findings are consistent with the secretion by cultured BAEC and RASM of both IGF I binding proteins (first peak), and IGF I (second peak). When IGF I release was quantitated by pooling fractions from the second peak, VSMC in culture consistently secreted more IGF I than endothelial cells (Figure 2).

Both endothelial and smooth muscle cells have been shown to possess specific high affinity receptors for IGF I. Because endothelial cells in particular have been shown to internalize IGF I and to retain it intact for significant periods of time, the possibility existed that IGF I release from these cells represented secretion of previously internalized growth factor. To demonstrate IGF I gene expression we performed Northern analysis of RNA extracted from
RASM and BAEC. Using a phosphorus-32–labeled rat IGF I cDNA probe that included the entire IGF I coding sequence, we detected barely visible transcripts in total RNA extracted from RASM. However, when RNA was enriched in polyadenylated RNA by two cycles of oligo(dT) cellulose chromatography, three primary IGF I mRNA transcripts were detected after hybridization and exposure of autoradiograms for 1–2 days (Figure 3). The sizes of these transcripts estimated from the positions of 28S and 18S ribosomal RNAs on the ethidium-bromide–stained gels were 8.2, 1.7, and 0.9–1.2 kb. To further provide evidence for IGF I gene expression in VSMC, solution hybridization/RNase protection assays were performed using a [32P]UTP-labeled antisense riboprobe containing the rat exon 3 coding sequence and flanking intron sequences. Hybridization of 40 μg total RNA from RASM to the riboprobe yielded the expected 182 bp–protected sequence after RNase digestion (Figure 4).

To document IGF I gene expression in endothelium, Northern hybridizations were performed using the 521 bp IGF I cDNA probe. Two primary transcripts were visible in polyadenylated RNA (A+) after high-stringency washings: a major transcript of 2.1 kb and a minor transcript of 1.6 kb (Figure 5). These transcripts were visible only after a long exposure of autoradiograms (10 days). No higher molecular weight IGF I mRNA transcripts were detected in endothelium. We could not perform RNase protection assays using endothelial RNA because the bovine IGF I cDNA has not been isolated.

FIGURE 3. Northern analysis of insulin-like growth factor I gene expression in rat aortic smooth muscle cells. Total RNA from confluent monolayers was enriched in polyadenylated RNA (A+) by two cycles of oligo(dT) cellulose chromatography. Ten micrograms A+ and A− RNA (i.e., RNA left after extraction of A+) were hybridized to a phosphorus-32–labeled IGF I complementary DNA probe. Positions of 28S and 18S ribosomal RNAs determined from the ethidium bromide–stained gel are shown. Three primary IGF I messenger RNA transcripts are shown and their sizes are 8.2, 1.7, and 0.9–1.2 kb.

FIGURE 4. Ribonuclease protection analysis of insulin-like growth factor I (IGF I) gene expression in rat aortic smooth muscle cells (RASM). Forty micrograms total RNA from RASM (lane 3) and of transfer RNA (lane 2) were hybridized to a single stranded IGF I exon 3 riboprobe, subjected to RNase digestion, and analyzed on a sequencing gel. Full-length undigested probe is shown in lane 1. A protected antisense RNA band of the predicted size is visible in lane 3. Sizes of bands were determined using a 5' end-labeled DNA ladder.

FIGURE 5. Northern analysis of insulin-like growth factor I (IGF I) gene expression in bovine aortic endothelial cells. Ten micrograms total RNA (T), A+ and A− RNA were hybridized to a phosphorus-32–labeled IGF I complementary DNA probe. Positions of 28S and 18S ribosomal RNAs are indicated. One major and one minor IGF I messenger RNA transcript are visible.
Discussion

IGF I is a ubiquitous growth factor that was traditionally believed to be produced mainly in the liver, although its secretion has been demonstrated from a variety of cultured cell types. The somatomedin "hypothesis" holds that one of the principle modes of action of growth hormone is to stimulate local tissue production of IGF I that then can act in an autocrine or paracrine manner to stimulate growth. The relative importance of circulating somatomedin of presumed hepatic origin, acting as a traditional endocrine growth stimulator as opposed to locally synthesized and regulated IGF I, has not been determined.

In the cardiovascular system, there has been little information about the synthesis or effects of IGF I. In vitro data indicate that IGF I stimulates growth of both endothelial as well as smooth muscle cells. In situ hybridization techniques have identified IGF I mRNA in epicardium and around coronary artery walls as well as low levels in neonatal rat cardiac myocytes. The resolution in these studies has not been sufficient to demonstrate specific cell types producing IGF I, in particular as regards endothelial cells and VSMC. A recent report has localized IGF I mRNA to the smooth muscle cell layer of rat aorta but not to endothelium. Our data provide evidence that endothelial cells and VSMC, the two primary cellular components of the arterial wall, synthesize and secrete IGF I.

Prior data have indicated that IGF I acts as a potent mitogen in platelet-derived growth factor (PDGF) to stimulate VSMC growth. PDGF appears to function essentially as a "competence" factor as defined by Stiles et al., who demonstrated that quiescent BALB/c-3T3 cells exposed to PDGF became competent to enter the cell cycle but that progression through G1 into S phase required the presence of "progression" factors such as IGF I. Likewise, in VSMC, IGF I appears to function essentially as a progression factor, and exposure of VSMC to PDGF in the absence of IGF I elicits only a weak mitogenic response. The possibility that VSMC may secrete their own progression factor was first raised by Clemmons et al., who demonstrated that exposure of VSMC to PDGF caused an increase in an IGF I-like peptide found in VSMC-conditioned medium. Our findings demonstrate IGF I gene expression in VSMC, and we have presented preliminary results indicating that PDGF regulates IGF I gene expression in RASM. These findings are consistent with an autocrine "loop" whereby VSMC may be induced to secrete their own progression activity under the influence of growth factors such as PDGF.

Intense IGF I immunostaining of both endothelial cells and VSMC is apparent in newly forming blood vessels, raising the possibility that it is fundamentally important in angiogenesis. This may be related to induction of IGF I gene expression in proliferating vascular cells or increased internalization of circulating or locally produced somatomedin. Because endothelium internalizes IGF I and retains it intact for extended periods of time in vitro, the endothelial cells may play a role in vascular cell growth in vivo through release of internalized circulating growth factor. However, our data are consistent with the possibility that IGF I gene expression and secretion is an intrinsic property of vascular endothelium and smooth muscle that is involved in local control of growth.

Changes in blood flow have been shown to induce endothelium-dependent remodeling of the vessel wall. Induction of a chronic increase in vascular load is associated with marked increases in IGF I immunostaining of both endothelial cells and VSMC in rat femoral artery. This raises the possibility that alterations in hemodynamic conditions regulate blood vessel wall IGF I content through increased uptake or local induction of IGF I gene expression. Alternatively, the increase in IGF I content may be secondary to arterial wall thickening.

The neointimal proliferative response after endothelial denudation of the rat femoral artery is accompanied by high levels of immunostainable IGF I in endothelial cells and VSMC. Recent data have indicated an increase in IGF I mRNA levels in rat aorta after endothelial denudation. Our data would support the hypothesis that endothelial cells and VSMC may be a primary source of IGF I in these injury models.

Several IGF I mRNA transcripts in various cell types and tissues have been identified by Northern analysis. The significance of these multiple transcripts, however, is not known. In VSMC, there are three primary IGF I transcripts, namely an 8.2, 1.7, and 0.9-1.2 kb species. We have occasionally identified a 3.9 kb transcript in VSMC, the significance of which is unknown. These transcript sizes are similar to previously reported transcripts for IGF I. In particular, rat aorta has been found to contain IGF I transcripts sized at 7.8 and 0.8 kb, and at 7.0, 1.8, and 1.0 kb. In striking contrast, however, endothelial cells demonstrate one major transcript of 2.1 kb and a minor transcript of 1.6 kb. It is possible that the 1.6 kb endothelial transcript and the 1.7 kb smooth muscle transcript represent the same message, but the 2.1 kb transcript may be unique for endothelium. Liver has been found to contain a 2.1 kb transcript that may well represent the contribution of RNA originating from endothelial cells. It is thus possible that some of the multiple IGF I transcripts described are cell type specific. Because the coding sequence for IGF I is highly conserved (95.7% homology between rat and human IGF I amino acid sequences and 100% homology between human and bovine IGF I amino acid sequences), we think that the lower abundance of message seen in endothelium is not due to the use of a heterologous probe.

In summary, our findings demonstrate the secretion of IGF I and IGF I binding protein activity by cultured BAEC and VSMC, and the expression of...
three primary IGF I mRNA transcripts in VSMC and one major as well as one minor IGF I mRNA transcript in endothelial cells. IGF I may play a central role in the endogenous growth program of the blood vessel wall, and regulation of IGF I gene expression in vascular tissues may be key to understanding developmental and abnormal growth in the cardiovascular system. Furthermore, it is possible that the paracrine effects of IGF I originating from blood vessels may be extended to adjoining nonvascular parenchymal cells. In this respect the capillary endothelium, by virtue of its tremendous surface area and its ubiquitous distribution, may serve to modulate tissue levels of IGF I and thus play a regulatory role in the control of organ growth. We are at present exploring these possibilities.

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