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Rat skeletal myoblasts and arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-*sis*) of platelet-derived growth factor (PDGF) and produce a PDGF-like protein

(autocrine stimulation/cell growth/differentiation/protooncogenes)

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ABSTRACT **It is shown here that the myogenic cell line L6J1, primary skeletal myoblasts, and primary adult arterial smooth muscle cells express the gene for the A chain but not the gene for the B chain (c-*sis*) of platelet-derived growth factor (PDGF). It is further demonstrated that conditioned media from L6J1 cultures contain material that (*i*) competes with ^{125}I-labeled PDGF for binding to human fibroblasts, (*ii*) is specifically precipitated by antibodies against PDGF, and (*iii*) has a relative molecular mass comparable to that of PDGF and, after reduction, its constituent subunit chains. The secretion of PDGF-receptor-competing activity was at a maximum in exponentially growing cultures but remained at a high level also after the cells had become confluent, stopped dividing, and fused to form multinucleate myotubes. Similarly, it was previously demonstrated that adult rat arterial smooth muscle cells in primary culture produce a mitogenic protein with immunological and structural properties similar to PDGF. In accordance with these findings, it was recently shown that secretion of PDGF-like mitogens by a number of human tumor cell lines correlates with expression of the gene for the A chain rather than the B chain of PDGF. The results suggest that production of homodimers of PDGF A chains may stimulate proliferation of skeletal myoblasts and arterial smooth muscle cells in an autocrine or paracrine manner. This could fulfill important functions during myogenesis in the embryo as well as in tissue repair and atherogenesis in the adult.**

Platelet-derived growth factor (PDGF) is a cationic, 30-kDa protein and a major mitogen for cells of mesenchymal origin. It is released during blood clotting, binds to specific receptors on the surface of the responding cells, and elicits a pleiotropic response that ultimately leads to DNA synthesis and mitosis (for a recent review, see ref. 1). Structurally, PDGF is a dimer of two polypeptide chains linked by disulfide bonds. Two similar but not identical types of subunit chains—named A and B—have been identified, but it is not known if PDGF is a heterodimer, a mixture of homodimers, or both.

The B chain of PDGF is encoded by the protooncogene c-*sis*, the precursor of the transforming gene of simian sarcoma virus (SSV), v-*sis* (2–6). SSV-infected cells produce a PDGF-like agonist, probably equivalent to a B–B-chain homodimer (7–11), and antibodies against PDGF have been found to inhibit transformation of cells by SSV (12). A number of tumor cell lines express c-*sis* and release PDGF-like mitogens (refs. 8, 13–18; for further examples, see ref. 19). However, the latter are not necessarily translation products of the c-*sis* gene. Thus, human osteosarcoma cells have been shown to secrete a growth factor structurally related to a homodimer of PDGF A chains (19). Moreover, it was recently demonstrated that the release of PDGF-like growth factors by a number of human tumor cell lines correlates with expression of the gene for the A chain rather than the B chain of PDGF (20). These findings suggest that endogenous PDGF-like growth factors of variable subunit composition may contribute to the transformed state, possibly by giving the cells a growth advantage.

Conceivably, autocrine or paracrine secretion of mitogens may be important also during normal growth and development and in other pathophysiological situations. The c-*sis* gene has been found to be expressed throughout mouse embryonic development (21). The cells of the cytotrophoblast layer of the human placenta express c-*sis* during the first trimester and explants of the placenta release PDGF-like activity into the culture medium (22). Endothelial cells (23, 24) and activated macrophages (25, 26) express c-*sis* and produce PDGF-like proteins but lack PDGF receptors. The role of the released factors may in these cases be to stimulate neighboring cells, for example, during wound healing. Arterial smooth muscle cells (SMC) from young (27) and adult (28) rats are two other examples of cells that have been found to produce PDGF-like mitogens. In adult SMC, the secretory activity peaks early in primary culture, shortly after modulation from contractile to synthetic state and commencement of rapid cell growth (28); the change in the phenotypic properties of the SMC represents a return to an earlier developmental stage (29, 30) and is *in vivo* an important early event in the formation of atherosclerotic lesions (31). The observations raise the possibility that autocrine secretion of PDGF-like molecules may stimulate SMC proliferation not only during embryonic and postnatal growth of large arteries but also during atherogenesis.

In the present investigation, it is demonstrated that skeletal myoblasts, multinucleate myotubes, and adult arterial SMC in primary culture express the gene for the A chain but not the gene for the B chain (c-*sis*) of PDGF. It is further shown that skeletal myoblasts actively secrete a 31-kDa protein that competes with PDGF for receptor binding and is precipitated by PDGF antibodies. The production of this protein decreased as the cells fused to form multinucleate myotubes and stopped dividing but nevertheless remained at a high level. The results are discussed with regard to a possible role

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Abbreviations: PDGF, platelet-derived growth factor; SMC, smooth muscle cells; SSV, simian sarcoma virus; kb, kilobase(s).

of autocrine growth stimulation in development of skeletal and smooth muscle, tissue repair, and atherogenesis.

MATERIALS AND METHODS

Cell Culture. Myoblasts of the cell line L6J1 (32) were seeded at 6×10^3 cells per cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum, penicillin (20 μg/ml), and streptomycin (20 μg/ml). For RNA analysis of terminally differentiated cultures, L6J1 cells were cultured for 11 days in DMEM/5% fetal calf serum without replating. Primary rat myoblasts were isolated from thighs of 18- to 20-day-old Sprague–Dawley rat embryos. The thigh muscles were minced by scissoring, trypsinized, and plated at 6×10^4 cells per cm^2 in DMEM with 10% horse serum and 1% embryo extract. SMC were isolated from the aortic media of adult (4- to 5-month-old) male Sprague–Dawley rats by digestion with 0.1% collagenase (Sigma, type I), seeded at 2×10^5 cells per cm^2, and cultured in Ham's F-12 medium with organic buffers (10 mM Hepes/10 mM Tes, pH 7.3), L-ascorbic acid (50 μg/ml), gentamycin sulfate (50 μg/ml), and 10% newborn calf serum (30). For isolation of RNA, primary rat myoblasts and arterial SMC were harvested in an active proliferative phase, about 7–14 days after plating.

Isolation and Blot Analysis of Poly(A)$^+$ RNA. One to 5×10^8 cells were harvested by scraping with a rubber policeman and RNA was isolated by hot phenol extraction (33). Total RNA was selected for poly(A)$^+$ RNA by chromatography on oligo(dT)-cellulose (34). Samples of the poly(A)$^+$ RNA were analyzed by ultraviolet spectrophotometry, fractionated through formaldehyde/agarose (1.1%) gels (35), transferred to nitrocellulose filters (36), and hybridized to cDNA probes nick-translated in the presence of [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) to specific activities of about 4×10^8 dpm/μg (36). Illustrated blots were washed in 15 mM NaCl/1.5 mM sodium citrate at 60°C. The sizes of the transcripts were determined relative to mouse 18S and 28S RNA. The following cloned DNA fragments were used as probes: human c-*sis*, pSM1 (37); human PDGF A chain, clone D1 (20); and mouse actin, pAM91 (38).

Southern Blot Analysis. High molecular weight rat L6J1 DNA was cleaved with *Eco*RI, separated by agarose (0.8%) gel electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled cDNA probes. The sizes of the hybridizing DNA fragments were determined relative to *Hin*dIII-cleaved λ DNA.

Conditioned Media. One or 8 days after plating, cultures of L6J1 cells were rinsed three times in phosphate-buffered saline (pH 7.3) and incubated for 48 hr with 80 μl of DMEM/5% fetal calf serum or Ham's F-12 medium per cm^2 with bovine serum albumin (0.5 mg/ml), Pederson's fetuin (10 μM), dexamethasone (0.1 μM), and insulin (1 μM). Prior to collection, another 0.5 mg of albumin per ml was added to the F-12 medium. The conditioned media were centrifuged at $200 \times g$ for 10 min, frozen, and stored at −20°C before further analysis.

Assay for PDGF Receptor-Competing Activity. The ability of the conditioned media to compete with ^{125}I-labeled PDGF (^{125}I-PDGF) for binding to human foreskin fibroblasts was measured as described (39). From a standard curve constructed with pure unlabeled PDGF (5–20 ng/ml), the PDGF receptor-competing activity of the samples was converted into PDGF equivalents (ng/ml).

Immunoprecipitation. A confluent 850-cm^2 roller bottle culture of L6J1 cells was incubated with 4 ml of cysteine-free Eagle's minimal essential medium containing 0.5 mCi of [^{35}S]cysteine (600 Ci/mmol, New England Nuclear) for 3 hr followed by a 1-hr chase in 4 ml of cysteine-containing medium. Labeling and chase media were pooled, clarified by centrifugation, and sequentially immunoprecipitated with nonimmune rabbit serum and anti-PDGF antiserum (40). The immunoprecipitates were then analyzed by NaDodSO$_4$/PAGE (41) and fluorography.

RESULTS

Expression of PDGF mRNA. The results of this (see below) and earlier studies (28) indicate that rat skeletal myoblasts of the established cell line L6J1 and adult rat arterial SMC in primary culture both produce PDGF-like factors. To determine which of the PDGF genes is expressed in these cell types, as well as in primary rat skeletal myoblasts, poly(A)$^+$ RNA was analyzed for the presence of PDGF A-chain and B-chain (c-*sis*) transcripts. The myogenic capacity of L6J1 cells and primary rat skeletal myoblasts under the *in vitro* conditions employed here has been described (42). Fig. 1 (*Lower*) shows that the nonmuscle 2.1-kilobase (kb) β- and γ-actin mRNA dominates in exponentially proliferating L6J1 myoblasts (lane 2) and that there is an increase in the muscle-specific 1.6-kb α-actin mRNA and a concomitant decrease of nonmuscle actin mRNA in terminally differentiated L6J1 myotubes (lane 3). The mixture of actin forms expressed in primary rat myoblasts (lane 5) indicates that partial differentiation has taken place, although no myotubes had yet formed at the time of cell harvest.

The RNA transfer blot analysis illustrated in Fig. 1 (*Upper*) shows that PDGF A-chain mRNA occurs in rat skeletal myoblasts and arterial SMC. A hybridizing band corresponding to a transcript of 1.7 kb was detected in all three cell types tested and an additional 2.3-kb mRNA was found in L6J1 myoblasts. Most likely, the 1.7- and 2.3-kb bands in the rat cells correspond to the 1.9- and 2.3-kb transcripts detected in a number of human tumor cell lines (20). A main difference between the rat smooth and skeletal muscle cells and the human tumor cells is that a 2.8-kb PDGF A-chain mRNA is expressed only in the latter. Densitometer scanning of the RNA transfer blot autoradiograms indicated that the level of the 1.7-kb PDGF A-chain transcripts in the mRNA preparations was about two times higher in the myoblasts than in the myotubes of the L6J1 cultures.

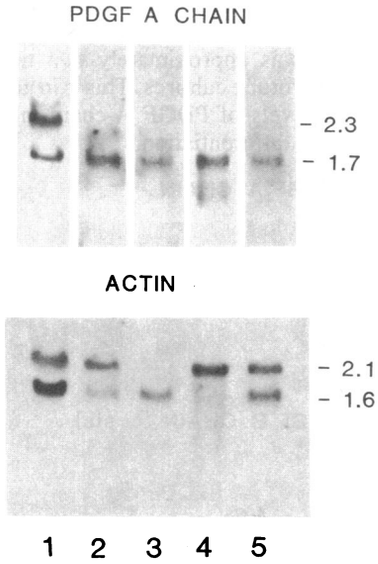

FIG. 1. RNA transfer blots illustrating PDGF A-chain transcripts in rat myogenic cells (*Upper*). Twenty micrograms of poly(A)$^+$ RNA from confluent L6J1 cells (lane 1) and proliferating arterial SMC (lane 4) and 10 μg of poly(A)$^+$ RNA from proliferating L6J1 myoblasts (lane 2), L6J1 myotubes (lane 3), and primary rat myoblasts (lane 5) were analyzed. The exposure time was 24 hr for lanes 1 and 4 and 4 days for lanes 2, 3, and 5. The expression of actin transcripts in the same material is also shown (*Lower*). Sizes are shown in kb.

After washing away the radioactively labeled A-chain probe, the nitrocellulose filters were rehybridized with a human c-*sis* probe. Even at low stringency conditions, no complementary transcripts were detected in any of the mRNA preparations, although the rat genome does contain DNA sequences that hybridize to a human c-*sis* probe at high stringency conditions (15 mM NaCl/1.5 mM sodium citrate, 60°C; Fig. 2). Since PDGF A-chain gene transcripts were redetected at unaltered intensity after the c-*sis* hybridization, the failure to detect c-*sis* transcripts cannot have been due to loss of RNA from the blots during washing.

The presence of a single PDGF A-chain hybridizing *Eco*RI band in the DNA (Fig. 2) suggests that the existence of A-chain mRNAs of two different sizes in L6J1 myoblasts is due to differential splicing rather than the existence of two genes.

Production of PDGF-Like Protein. Serum-free conditioned medium from adult rat arterial SMC in primary culture was recently shown to contain a PDGF-like protein. Its mitogenic activity was neutralized by antibodies against PDGF and partially purified material from the conditioned medium competed with ^{125}I-PDGF for receptor binding in a dose-dependent manner (28). To investigate whether rat skeletal muscle cells also produce a PDGF-like factor, we have analyzed conditioned media from cultures of the myogenic cell line L6J1 for PDGF receptor-competing activity. Conditioning started 1 or 8 days after plating. At the early time point, the myoblasts grew exponentially as mononucleate cells. After 8 days, they had reached confluence and fused to form multinucleate myotubes; ≈80% of the nuclei were incorporated into myotubes.

PDGF receptor-competing activity was found in the media from proliferating myoblasts and from terminally differentiated myotubes (Fig. 3). In DMEM/5% fetal calf serum, the activities were equivalent to 22 and 38 ng of PDGF per ml, respectively. In F-12 medium, the corresponding values were lower, 10 and 23 ng/ml, respectively. Thus, in both media the PDGF-like activity was about twice as high in myotube as in myoblast cultures. At the same time, the number of cell nuclei was four times higher at the myotube stage. Calculated per nucleus, this means that the rate of production of the PDGF-like factor was approximately two times higher in myoblast than in myotube cultures. This is in good agreement with the relative levels of PDGF A-chain mRNA at these stages of myogenic differentiation.

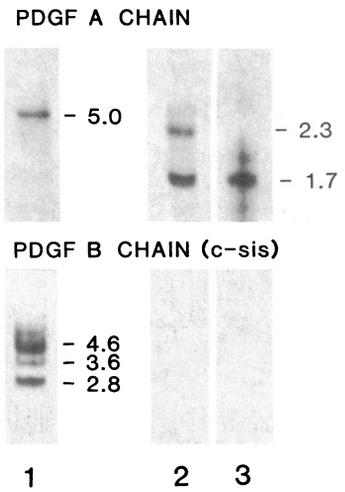

FIG. 2. DNA and RNA blot analysis of PDGF A chain (*Upper*) and c-*sis* (*Lower*). Lane 1, 10 μg of L6J1 DNA cleaved with *Eco*RI; lanes 2 and 3, 50 μg of poly(A)$^+$ RNA from L6J1 and arterial SMC, respectively. Sizes are shown in kb.

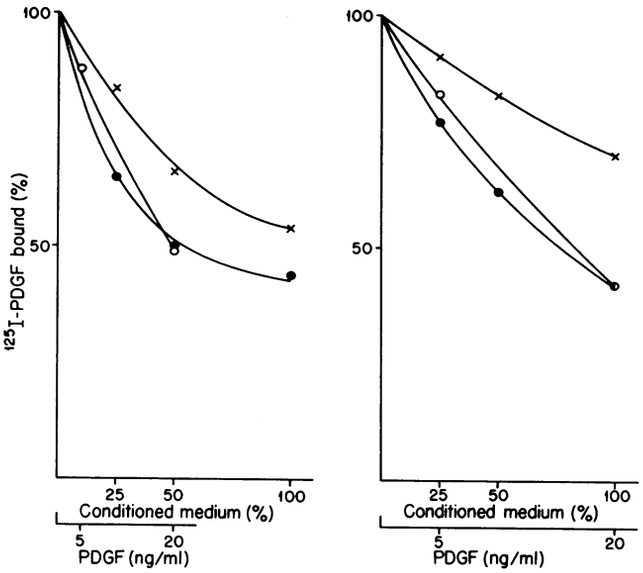

FIG. 3. Effect of conditioned media from L6J1 myoblast cultures and unlabeled PDGF on binding of ^{125}I-PDGF to human foreskin fibroblasts. L6J1 cells were cultured for 1 day (×) or 8 days (●) before the start of conditioning. ○, Unlabeled PDGF. (*Left*) Conditioned DMEM/5% fetal calf serum. (*Right*) Conditioned F-12 medium. Each value is the mean of triplicate cultures.

The PDGF-like nature of the material produced by the L6J1 cells was further demonstrated by immunoprecipitation of conditioned medium from cultures metabolically labeled with [^{35}S]cysteine. NaDodSO$_4$/PAGE showed that the PDGF antibodies specifically precipitated a protein that under nonreducing conditions migrated as a distinct 31-kDa band and after reduction dissociated into subunits of 17 kDa (Fig. 4).

DISCUSSION

Using a newly constructed cDNA probe (20), it is demonstrated that rat skeletal myoblasts and adult arterial SMC both express the gene for the A chain of PDGF. In contrast, no signs of expression of c-*sis*, the B-chain gene, were detected. It is also shown that rat skeletal myoblasts release a PDGF-like protein. The secretory activity continued at a reduced but highly significant level after the cells had become confluent, stopped dividing, and fused to form myotubes. The PDGF-like nature of the material produced by the cells

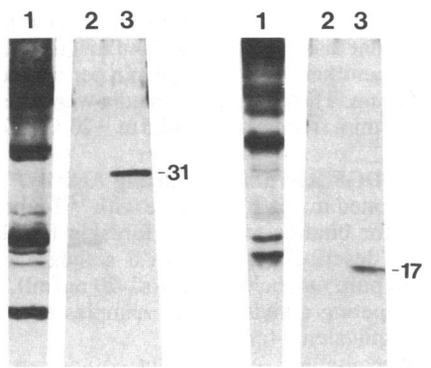

FIG. 4. Immunoprecipitation of metabolically labeled protein secreted by L6J1 myoblasts. The precipitated material was analyzed by NaDodSO$_4$/PAGE under nonreducing (*Left*) or reducing (*Right*) conditions. Lanes: 1, starting material; 2, precipitation with nonimmune serum; 3, precipitation with anti-PDGF antiserum. Sizes are shown in kDa.

was established on the basis of its ability to compete with authentic PDGF for binding to cell-surface receptors, precipitation of metabolically labeled molecules with antibodies against PDGF, and determination of relative molecular mass with and without prior reduction. Similarly, rat arterial SMC were recently demonstrated to produce a PDGF-like protein and stimulate their own growth in an autocrine manner (28). The secretion of the mitogen peaked during the most active growth phase in primary culture, decreased but did not entirely cease as confluence was reached, and then remained at a low level after trypsinization and replating in secondary culture. Taken together, the results suggest that the PDGF-like proteins secreted by the muscle cells consist of homodimers of A chains. This is supported by the immunoprecipitation data, which indicated that the product secreted by the L6J1 cells migrated in a similar manner to the osteosarcoma-derived growth factor, a PDGF A-chain homodimer (19), during $NaDodSO_4$/PAGE. Likewise, production of PDGF-like proteins by a number of human tumor cell lines was found to correlate with expression of A-chain but not B-chain mRNA (20).

In vivo production of PDGF by skeletal myoblasts and arterial SMC could be of physiological importance during the embryonic and postnatal periods as well as in repair processes in the adult. In developing skeletal muscle, the initial stages are characterized by proliferation of myoblasts. The observations of the present investigation suggest that autocrine production of PDGF may be involved in this process. Earlier studies have shown that proliferating myoblasts also express the protooncogenes c-*ras*, c-*abl*, and c-*myc* (42). Whereas expression of these protooncogenes markedly decreased as the cells became confluent, stopped dividing, and fused to form myotubes, the production of PDGF continued at a moderately reduced level. The functional role of the growth factor at this nonproliferative stage is unknown.

In developing arteries, the SMC are in a synthetic state distinguished by rapid proliferation and active production of extracellular matrix components, two functions known to be stimulated by PDGF (29). Autocrine production of this factor could therefore be a means to promote arterial growth in the evolving organism. In support of this idea, SMC from young rat aortas were found to secrete PDGF-like protein in a stable manner during repeated passages in culture (27). In the adult, arterial SMC are in a contractile, nonproliferative state (29, 30). During atherogenesis, they revert to a synthetic state and start again to divide and deposit extracellular matrix (31). In an analogous way, adult arterial SMC modulate from contractile to synthetic state during *in vitro* cultivation but do not synthesize DNA and divide unless exposed to PDGF or other growth factors (29, 30). However, after a few divisions the need for exogenous mitogens decreases and autocrine production of PDGF-like protein starts (28, 30). As shown here, this is associated with expression of the gene for the A chain of PDGF.

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