Type β Transforming Growth Factor in Human Platelets: Release during Platelet Degranulation and Action on Vascular Smooth Muscle Cells

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Abstract. A specific radioimmunoassay for type beta transforming growth factor (TGF-β) was developed and used to show that human platelets treated with thrombin release TGF-β as a consequence of degranulation. The thrombin concentrations required to induce release of TGF-β parallel those concentrations that release the alpha-granule marker, beta-thromboglobulin. Related studies showed that TGF-β acts on early passage, explant cultures of bovine aortic smooth muscle cells by inhibiting the effect of mitogens on proliferation of subconfluent cell monolayers yet synergizing with mitogens to stimulate growth of the same cells when cultured in soft agar. The results show that primary cultures of bovine aortic smooth muscle cells and established normal rat kidney cells behave similarly with regard to TGF-β action. Moreover, the data suggest that platelet-mediated proliferation of aortic smooth muscle cells in vivo may not result solely from the stimulatory effect of platelet-derived growth factor (PDGF), but rather from an interaction of platelet factors which has the intrinsic ability to limit as well as stimulate mitosis.

THE rate of fibroblast and smooth muscle cell division is controlled, in large part, by the action of peptide growth factors present in platelets. Physiologically, these peptides are released as a consequence of platelet degranulation at sites of injury; together with growth factors in plasma, they stimulate mitosis of connective tissue cells (36, 37, 47). The best studied of these platelet peptides is platelet-derived growth factor (PDGF),[1] a component of alpha-granules (16, 17) that is a chemoattractant (13, 41, 42) and mitogen (36, 37, 47) for fibroblasts and vascular smooth muscle cells. It is important that PDGF has also been implicated in the etiology of proliferative diseases. Expression of PDGF-like proteins is a common feature of many transformed cells (7) and the hallmark of transformation by simian sarcoma virus (11, 52). Similarly, exposure of vascular smooth muscle cells to PDGF (35) or PDGF-like proteins (10, 40, 43) may be an important component in the proliferation of these cells during atherogenesis.

We and others have shown that PDGF is only one of several growth factors in human platelets (4–6, 9, 14, 26); type beta transforming growth factor (TGF-β) can be purified (5) from platelets with yields similar to that of PDGF. TGF-β is a widely distributed growth factor in animal tissues (27), but platelets contain ~100-fold more TGF-β than do the other cells or tissues examined to date (5). Consistent with the role of platelet factors as participants in wound repair, TGF-β can

stimulate cell proliferation, protein synthesis, and collagen production in an in vivo wound healing system (46). A functional, epidermal growth factor (EGF)–like peptide is also present in human platelets (4, 6, 26), but lack of the purified protein has precluded detailed studies of its structure and action.

The initial in vitro studies with TGF-β focused on its role in cellular transformation (28, 30); this peptide stimulates growth of non-neoplastic fibroblastic cells suspended in medium with 10% serum and agar (conditions of anchorage-independent growth). In particular, NRK fibroblasts grow efficiently in soft agar when both TGF-β and EGF (or TGF-alpha, an EGF-like peptide) are added to the culture medium (1, 28, 30). AKR-2B fibroblasts require only the addition of TGF-β (50). Recent studies with TGF-β have demonstrated that this growth factor is actually a bifunctional regulator of cell proliferation; in addition to its stimulatory effects on anchorage-independent growth of mesenchymal cells, TGF-β can inhibit the soft agar growth of many epithelial tumor cells and several fibroblastic cell lines (29, 49). In NRK cells, TGF-β inhibits monolayer growth of subconfluent cultures yet stimulates the growth of confluent monolayers and cells suspended in agar (29).

The inhibitory effects of TGF-β on monolayer cultures of NRK cells have not been observed in all studies (21), suggesting that culture conditions can alter the cellular response to TGF-β. The widespread use of subcloned cell lines has further complicated the interpretation of TGF-β action. Moses et al. (24) have recently studied the effects of TGF-β on early

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[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; NRK, normal rat kidney; PDGF, platelet-derived growth factor; β-TGF, type β transforming growth factor.

passage skin fibroblasts in an attempt to examine the action of this growth factor in uncloned cell cultures. The results show that the growth factor requirement for colony formation of these primary fibroblasts resembles that of established AKR-2B cells; TGF-β stimulates colony formation in the absence of a detectable synergism with EGF.

The experiments reported here were designed to examine the potential role of TGF-β as a mediator of cell proliferation during wound repair and atherogenesis. We show that immunoreactive TGF-β is released from platelets during thrombin-induced degranulation and that the same concentrations of thrombin release TGF-β and the alpha-granule marker (16, 17) beta-thromboglobulin. We also show that TGF-β acts on explant cultures of bovine aortic smooth muscle cells by inhibiting growth when the cells are cultured as subconfluent monolayers and synergizing with EGF and PDGF to stimulate growth when the cells are cultured in soft agar. The results suggest that release of TGF-β during platelet degranulation may be an important element in the regulation of smooth muscle cell division.

Materials and Methods

Growth Factors

TGF-β was purified from freshly drawn human platelets as described (5) except that urea was removed from the purified protein by desalting with high pressure liquid chromatography (2). EGF was purified from mouse submaxillary glands similarly to described procedures (39). Final preparations migrated as single bands during SDS PAGE, eluted as single peaks during analytical C-18 high pressure liquid chromatography, and had amino acid compositions in agreement with published values. Porcine PDGF (Bethesda Research Laboratories), was ~95% pure as determined by SDS gels and lacked TGF-β as determined by specific receptor binding assays. Human PDGF, purified to homogeneity, was generously provided by Gary Grotendorst and Russell Ross. TGF-β, EGF, and albumin-free samples of PDGF were quantitated by amino acid analysis. Growth factor–enriched extracts of human platelets were prepared as described (5), freeze-dried, redissolved in 4 mM HCl, and neutralized with protein-free culture medium. Protein concentration of the extract was determined by the Coomassie binding procedure (Bio-Rad Laboratories, Richmond, CA) using bovine plasma albumin as reference. All growth factor solutions were diluted into culture medium (see below) containing 1 mg/ml bovine serum albumin before use. Neutralized solutions of PDGF and platelet extract were heated (100°C, 5–10 min) before use.

Radioimmunoassay for TGF-β

Rabbit antiserum (N-113), prepared against keyhole limpet hemocyanin–coupled TGF-β from human platelets, was titered by its ability to bind ^{125}I-labeled TGF-β (prepared as described in reference 12) and used for radioimmunoassay in an amount (0.5 μl/tube) that precipitated 50% of the bindable tracer. The titration was performed in siliconized Eppendorf tubes (1.5-ml capacity) by incubating (2 h at room temperature) selected volumes of antiserum or normal rabbit serum (both diluted to 125 μl with buffer) with ^{125}I-labeled TGF-β (10^4 cpm in 25 μl) and precipitating the bound label (1 h at room temperature with gentle rocking) with fixed *Staphylococcus aureus* cells, (Boehringer-Mannheim Diagnostics, Houston, TX; 25 μl diluted with 1 ml buffer). Precipitated radioactivity was collected by high-speed centrifugation (1 min, table-top centrifuge, Brinkmann Instruments Co., Westbury, NY), the supernatant was carefully aspirated, and the tube with the pellet was counted for radioactivity. The radioimmunoassay was performed under similar conditions except that selected amounts of unlabeled TGF-β from human platelets (diluted to 0.1 ml with buffer) were incubated (2 h at room temperature) with antiserum (0.5 μl in 25 μl buffer) before the addition of labeled TGF-β. The buffer for both titration and radioimmunoassay was a solution of 0.1 M Tris, 0.5% Nonidet P-40, 0.1% SDS, 0.15 M NaCl that had been brought to pH 8.0 with HCl and then supplemented with crystalline bovine serum albumin and aprotinin solution (2% of each as final concentrations; Sigma Chemical Co., St. Louis, MO). The *S. aureus* cells were washed three times (by repeated suspension and centrifugation) with a 10-fold excess of buffer per wash, and suspended to half the

original volume before use. Data are plotted as means of duplicate determinations. Each data point usually varied from its mean by <5%.

Platelet Release Reaction

Human platelet–rich plasma (4 ml, Community Blood and Plasma, Baltimore, MD) was purified by gel filtration over a siliconized column (1.5 × 30 cm) of Sepharose 2B equilibrated in calcium-free Tyrode's solution (18) and run at a flow rate of 15–25 ml/h. The gel-filtered platelets were collected in 1-ml fractions, quantitated with a Coulter counter, and suspended (10^8/ml) in calcium-free Tyrode's solution. Aliquots (0.9 ml) of the suspension were incubated with gentle mixing (in siliconized test tubes) in a 37°C water bath for 5 min before the addition of 0.1 ml of a thrombin solution (diluted in calcium-free Tyrode's soultion, 37°C, 0–1 U/ml final concentration; Sigma Chemical Co.). After 3 min with thrombin, the supernatant (containing released platelet factors) was collected by centrifugation. Careful collection of the top 80–90% of the supernatant prevented contamination of the sample with platelets.

Cell Cultures

NRK fibroblasts (clone 49F) were maintained in Dulbecco's modified Eagle's medium containing gentamicin (DME) and 5% fetal bovine serum (Bio-Fluids Inc., Rockville, MD). Bovine vascular smooth muscle cells were obtained by explant culture from the medial layer of fetal bovine aorta as described (13, 34). In brief, the explants were cultured in T-75 flasks with DME, 10% in fetal calf serum. The cultures were fed biweekly and trypsinized after 3–4 wk. The trypsinized cultures were suspended in medium and filtered through four layers of sterile gauze to remove the small pieces of medial aorta. The resulting preparations of smooth muscle cells (defined as passage 1) were placed in two T-75 tissue culture flasks, maintained in medium with 5% fetal bovine serum, and thereafter split in a 2:1 ratio. Smooth muscle cells used in the experiments reported here were at passages 2–4.

Assays for Cell Growth

Cell growth was measured in both soft agar and monolayer culture. The soft agar assay was performed as described (5, 31) with 3,000 cells seeded in DME containing 10% calf (whole blood-derived) serum (Gibco, Grand Island, NY) or 10% bovine plasma-derived serum. (Bovine plasma was prepared at the National Institutes of Health-Poolesville Animal Center by plasmaphoresis of 4–12-mo-old calves. The plasma was sterilized by filtration and converted to 10% plasma-derived serum in DME as described in reference 4.) To measure mitosis of NRK and smooth muscle cells in monolayer culture, 1.4-ml portions of a cell suspension (3,000 cells/ml) were added to tissue culture dishes (35 × 100 mm; resulting monolayers were ~10% confluent) and incubated for 8 d. At selected intervals the cultures were trypsinized, and the number of cells was determined with a Coulter counter. The medium for monolayer cultures was DME with 5% plasma-derived serum; 5% fetal calf serum; or 5% plasma-derived serum containing 0.1 nM EGF. In some experiments, TGF-β (20 pM) was added to the cultures 4–6 h after seeding. Stimulation of DNA synthesis by PDGF was determined with both smooth muscle and NRK cells cultured in 1% plasma-derived serum as described (3) except that ^{125}I-deoxyuridine (Amersham Corp., Arlington Heights, IL 1 μCi/well) was used instead of [^{3}H]-thymidine. In some experiments, the assay was modified to examine stimulation of DNA synthesis by EGF and TGF-β (see the legend to Table I for details).

Results

Initial experiments were designed to study the release of TGF-β during platelet degranulation. A radioimmunoassay, rather than a bioassay, was used to examine the released products for TGF-β, thus minimizing the likelihood that other released platelet factors, such as PDGF (16, 17), might interfere with TGF-β quantitation. Fig. 1 shows the characteristics of the radioimmunoassay developed for TGF-β. Antiserum (N-113), generated in rabbits against keyhole limpet hemocyanin–coupled TGF-β from human platelets, effectively bound the radioiodinated growth factor: 50% of maximal immunoprecipitation occurred with 0.5 μl of the unfractionated, heat-inactivated serum (Fig. 1, inset, solid circles). This amount of antiserum was used under conditions of radioimmunoassay (Fig. 1) and detected ~0.5 ng unlabeled TGF-β. PDGF (0.1–
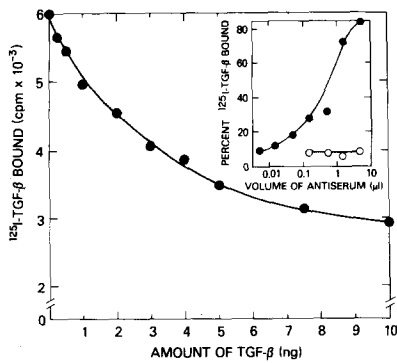
Figure 1. Radioimmunoassay for TGF-β. The figure shows specific binding of radiolabeled TGF-β to antiserum N-113 and use of the antiserum under conditions of radioimmunoassay. ●, antiserum. ○, normal rabbit serum. See Materials and Methods for protocols.
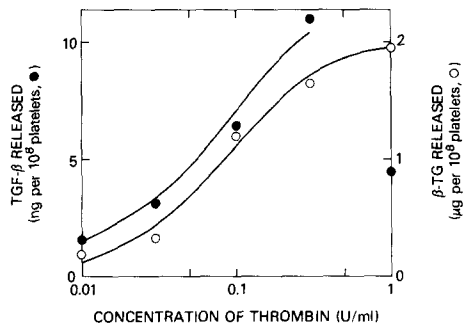

Figure 2. Release of TGF-β and beta-thromboglobulin by treatment of human platelets with thrombin. Gel-filtered human platelets were degranulated by exposure to thrombin (see Materials and Methods). The supernatants were tested for presence of beta-thromboglobulin (○) and TGF-β (●) using a commercial radioimmunoassay kit (Amersham Corp.) and the assay described in Fig. 1, respectively. TGF-β detection required concentration of the supernatant (0.5-ml portions of the supernatants were dialyzed against 1 M acetic acid in a microdialysis unit [Bethesda Research Laboratories], freeze-dried, and dissolved in 0.1 ml of radioimmunoassay buffer (see legend to Fig. 1) modified such that the final albumin concentration was 20 mg/ml). When this entire sample was assayed (corresponding to released products from 0.5 × 10⁸ platelets), immunoreactive TGF-β was detected at 0.8–5 ng (for 0.01–0.3 U of thrombin, respectively). Immunoreactive beta-thromboglobulin was detected at 20 to 100 ng after a 10- to 20-fold dilution of the supernatants into radioimmunoassay buffer. The basis for the apparent decrease in release of TGF-β with 1 U/ml thrombin is not clear at present.

100 ng), EGF (0.1–1,000 ng), and insulin (0.1–1,000 ng) were not detected at all by the immunoassay (data not shown). Others have shown that this antiserum also neutralizes the activity of TGF-β in bioassays (23).

Fig. 2 shows the use of the immunoassay to examine release of TGF-β from fresh gel-filtered human platelets after their degranulation with thrombin. The results show that thrombin treatment efficiently releases immunoreactive TGF-β (solid circles). Moreover, the concentrations of thrombin that release the growth factor agree well with those concentrations that release the alpha-granule marker (16, 17) beta-thromboglobulin (open circles). Quantitative analysis of the data in Fig. 2 indicates that a single human platelet releases 1,200–2,400 molecules of TGF-β. A similar value (2,500 molecules TGF-β per platelet) can be derived from the yield of the growth
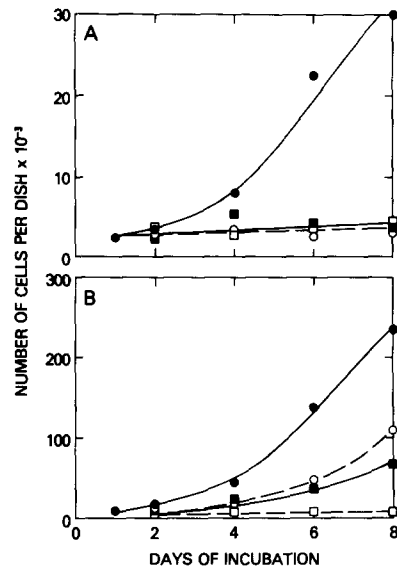

Figure 3. Effect of TGF-β on growth of smooth muscle and NRK cells in monolayer. Sparse monolayers of smooth muscle cells (*A*) and NRK cells (*B*) were prepared as described in Materials and Methods and cultured in DME containing 5% plasma-derived serum (squares) or 5% plasma-derived serum, 0.1 nM in EGF (circles). The experiment was performed in the presence (open symbols) and absence (filled symbols) of TGF-β (20 pM).

factor after purification (assuming 20% recovery of TGF-β [2, 5]), demonstrating that platelet degranulation releases a large part of intracellular TGF-β. These results support other studies showing that TGF-β stimulates protein synthesis and cell division in a rat wound healing system (46).

In addition to the physiological release of PDGFs during wound repair, these peptides may have an important role in atherogenesis; other investigators have suggested that exposure to PDGF, or PDGF-like molecules, may be an important component in the proliferation of aortic smooth muscle cells (35, 43). We have examined the effect of TGF-β on monolayer and soft agar growth of explant cultures from bovine aortic smooth muscle.

As expected from the work of others (36, 37), sparse monolayers of these cells (Fig. 3*A*) became quiescent when maintained in medium containing 5% plasma-derived serum (solid squares) and divided regularly when the medium contained 5% whole blood–derived serum (data not shown) or 5% plasma-derived serum, 0.1 nM in EGF (solid circles). The addition of TGF-β did not stimulate growth of the quiescent cells (open squares) and antagonized the effect of EGF in the growing cultures (open circles). Control studies (data not shown) demonstrated that TGF-β also inhibited the effect of 5% calf serum on smooth muscle cell growth in monolayer. Interestingly, these effects of TGF-β on vascular smooth muscle cells are strikingly similar to those observed for the monolayer growth of established NRK fibroblasts studied in parallel and under identical conditions (Fig. 3*B* and reference 29).

The similar effects of TGF-β on monolayer growth of vascular smooth muscle and NRK cells was confirmed in a mitogen assay measuring [³H]thymidine incorporation after incubation of growth factors for 22 h with confluent serum-starved cells (Table I). TGF-β did not stimulate incorporation

Table I. Effect of EGF, Serum, and TFG-β on [³H]-Thymidine Incorporation in Monolayer Cell Cultures

| | [³H]Thymidine incorporation (cpm × 10⁻³/well) | | | |
| | Smooth muscle | | NRK | |
Growth factor	22 h	42 h	22 h	42 h
None	4	4	50	40
EGF (0.1 nM)	12	ND	190	ND
Calf serum (5%)	100	70	250	166
TGF-β				
1 pM	5	3	47	45
10 pM	4	2	33	29
100 pM	4	3	14	42

The table shows the ability of EGF, calf serum, and TGF-β to stimulate [³H]-thymidine incorporation into DNA of serum-starved confluent cells. The experiment was performed similarly to the procedure described (5). In brief, cells were seeded in 96-well plates in DME, 5% calf serum. The next day, the medium was changed to DME, 0.2% calf serum. Cells were incubated in this low-serum medium for 3 d, during which they became confluent and quiescent. Growth factors were incubated with the quiescent cells for 15 to 35 h before the addition of [³H]thymidine. After a 7-h pulse with the radiolabel (22 or 42 h total incubation with growth factor), the cells were fixed with 5% trichloroacetic acid, washed, extracted, and counted for radioactivity. EGF and TGF-β were tested at concentrations active in both monolayer (see Fig. 3) and soft agar (see Fig. 4) cultures. EGF was tested under standard mitogen assay conditions for mesenchymal cells (about 22-h prereplicative phase), whereas as TGF-β was tested under both standard and prolonged (42 h prereplicative phase) assay conditions. Calf serum (5%) was considered the positive control under both conditions. ND, not determined.

of [³H]thymidine in this system despite the fact that EGF and calf serum were effective mitogens. Shipley et al. (44) have reported that in quiescent AKR-2B cells, TGF-β stimulates mitosis after a prolonged prereplicative phase of 30–42 h. We have examined the effects of TGF-β on [³H]thymidine incorporation under similar conditions (Table I, 42 h), but, again, could not detect an overall stimulation of DNA synthesis in smooth muscle or NRK cells. These results distinguish the effects of TGF-β in smooth muscle and NRK cells from those in AKR-2B cells. Massagué (21) has reported that TGF-β stimulates mitosis of sparse, serum-starved NRK cells. We perform mitogen assays with confluent cultures, but our results with [³H]thymidine incorporation agree with the effect of TGF-β on the long-term monolayer growth of subconfluent cells cultured in plasma-derived serum (see Fig. 3).

When bovine aortic smooth muscle cells are suspended in medium containing 10% serum and agar, their rate of growth (colony formation) is stimulated, rather than inhibited, by the addition of TGF-β (Fig. 4A, solid circles). In this culture system, the effect of TGF-β is only observed when both EGF and PDGF are also added; absence of exogenous EGF (Fig. 4A, open circles and Fig. 4B) or PDGF (Fig. 4A, open triangles) decreases colony formation 5- or 10-fold, respectively. Again, these growth factor effects on colony formation of smooth muscle cells resemble those for NRK fibroblasts, especially with regard to the EGF dependency of colony formation (compare Fig. 4B with references 1, 4, 28, 30). Comparative dose response curves for the growth factors used by NRK and smooth muscle cells in soft agar showed that colony formation in the two cell types is induced by similar concentrations of TGF-β and EGF. However, the amount of PDGF required to induce colony formation of smooth muscle cells exceeds that required by NRK fibroblasts. In NRK cells, the need for PDGF is satisfied by culturing the cells in soft agar containing 10% serum; PDGF must be added to this
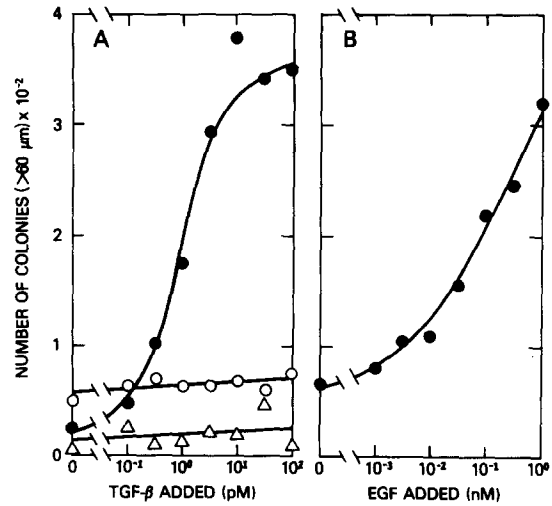

Figure 4. Effect of TGF-β on growth of smooth muscle cells in soft agar. The assay was performed as described in Materials and Methods in medium containing 10% calf serum. For the experiment shown in A, the assay was supplemented with 1 nM PDGF and 0.5 nM EGF (●), 1 nM PDGF (○), or 0.5 nM EGF (△). For the experiment shown in B, the assay was supplemented with 1 nM PDGF and 20 pM TGF-β.
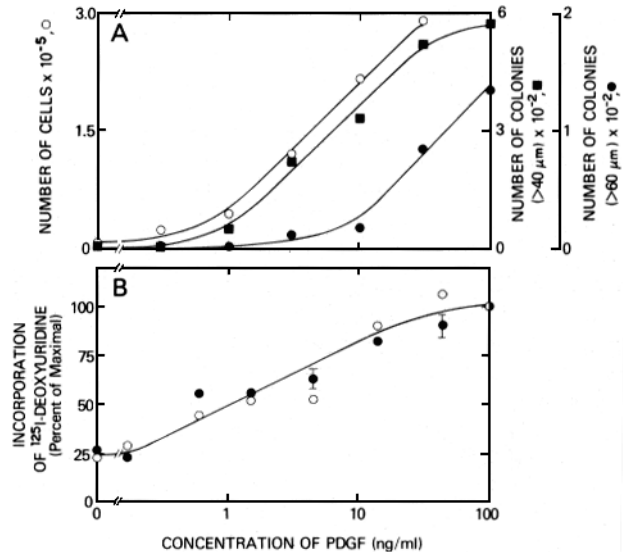

Figure 5. Ability of purified PDGF to stimulate monolayer and soft agar growth of smooth muscle cells. A shows the concentrations of purified PDGF required to stimulate growth of smooth muscle cells cultured in monolayer (○) and soft agar (■, ●). The assays were performed as described in Materials and Methods except that the medium for both culture conditions was DME with 10% plasma-derived serum. The soft agar cultures were supplemented with TGF-β (20 pM) and EGF (0.5 nM) in order to elicit the PDGF effect. Both assays were incubated at 37°C for 7 d. B shows the ability of purified PDGF to stimulate ¹²⁵I-deoxyuridine incorporation into trichloroacetic acid–insoluble products in smooth muscle (○) and NRK (●) cells. Basal ¹²⁵I-deoxyuridine incorporation was 1,700 and 7,250 cpm for smooth muscle and NRK cells, respectively. Maximal stimulation for PDGF was defined as the response of cells to 100 ng/ml of the purified growth factor.

system only when the cells are grown in 10% plasma (4). In contrast, colony formation of early passage bovine smooth muscle cells cultured in 10% calf serum (Fig. 4A) still requires

the addition of PDGF.

The experiments shown in Fig. 5 examined the PDGF requirement of the smooth muscle cells in detail. For the results shown in Fig. 5A, cells were grown in soft agar containing 10% plasma-derived serum (rather than whole blood–derived serum) in order to decrease the background levels of platelet-derived peptides. Under these conditions, overall colony formation (colonies of >40 μm diam, ~10 cells, solid squares) is induced (in the presence of saturating amounts of TGF-β and EGF) with concentrations of PDGF that are just slightly greater than those that stimulate cell growth in monolayer (Fig. 5, open circles). Efficient cell division in soft agar (formation of colonies greater than 60 microns in diameter, solid circles) requires about 10-fold more PDGF than does division in monolayer. Identical results were obtained with each of the three PDGF preparations described in Materials and Methods. Note that stimulation of smooth muscle cell division in monolayer (open circles) is induced by the same concentrations of PDGF that are active in other systems (4, 15, 38, 53), and that smooth muscle and NRK cells respond equivalently to PDGF in a monolayer assay measuring stimulation of DNA synthesis (Fig. 5B). Thus, the high concentration of PDGF required to induce colony formation of smooth muscle cells does not reflect an overall insensitivity to this growth factor.

Despite the fact that the PDGF preparations used in these studies were purified to homogeneity by distinct methodologies (32, 48) and exhaustively heated (100°C, 5–10 min) before use, we have considered the possibility that stimulation of smooth muscle cell growth in soft agar by high concentrations of PDGF might be due to heat-stable contaminants in the preparations rather than to PDGF itself. If small amounts of contaminating platelet factors were responsible for induction of colony formation in these preparations of purified PDGF, then crude PDGF should stimulate soft agar growth of smooth muscle cells more efficiently. We have used a heated platelet extract as a source of highly impure PDGF and show (Fig. 6) that colony formation of vascular smooth muscle cells (assayed in the presence of saturating concentrations of TGF-β and EGF, solid symbols) still requires more PDGF than does stimulation of DNA synthesis in monolayer (open circles). Moreover, the quantitative relationship between the amount of PDGF that stimulates DNA synthesis in monolayer, small
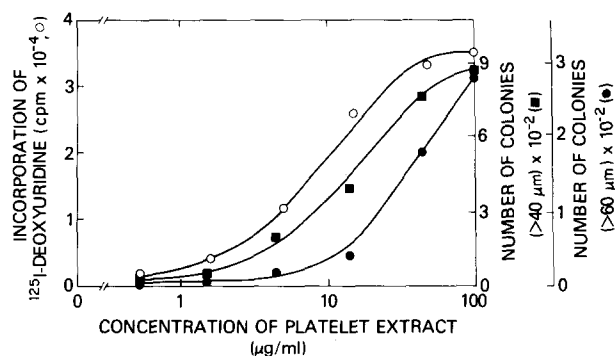

Figure 6. Ability of a crude platelet extract to stimulate monolayer and soft agar growth of smooth muscle cells. The figure shows the concentrations of a heated platelet extract (see Materials and Methods) that stimulates ^{125}I-deoxyuridine incorporation in monolayer (○) and colony formation in soft agar (■, ●). See the legend to Fig. 5 for details of the soft agar and monolayer assays.

colony formation, and large colony formation is identical for both pure and crude PDGF (compare Figs. 5 and 6; open circles, filled squares, and closed circles, respectively). We conclude that high concentrations of PDGF itself are required to synergize with TGF-β and EGF and stimulate colony formation of bovine aortic smooth muscle cells.

Discussion

We have developed a specific radioimmunoassay for TGF-β and shown that this growth factor is released from platelets degranulated with thrombin. Note that the concentrations of thrombin that release TGF-β also release the alpha-granule marker beta-thromboglobulin. These results suggest that TGF-β, like PDGF (16, 17), is contained within the platelet alpha-granule, but a direct analysis of TGF-β content in isolated alpha-granules remains an important matter for further study. Similarly, recent studies have shown that certain cell lines secrete TGF-β in a biologically inactive state (8, 20); the activity can be expressed in vitro by exposure of the growth factor to acid (pH 2). We are currently examining the biological state of TGF-β in platelets.

In addition to the generally accepted role of PDGFs in wound repair, Ross and Glomset have suggested that exposure of aortic smooth muscle cells to PDGF may stimulate proliferation of smooth muscle cells in atherogenesis (35). In vivo model systems examining the effect of platelet adhesion and degranulation on proliferation of aortic smooth muscle cells have yielded conflicting results (34, 45), but it is clear that aortic smooth muscle cells in culture do proliferate in response to PDGF. The data reported here show that platelet-derived TGF-β also acts on cultures of aortic smooth muscle cells. The effect of TGF-β on these cells depends on conditions of cell morphology and culture; TGF-β inhibits proliferation of subconfluent monolayers yet stimulates EGF-dependent growth of the cells when suspended in soft agar. These effects are qualitatively identical to those observed with NRK cells (reference 29 and this report) and thereby identify a primary cell that behaves similarly to established NRK cells with regard to TGF-β action. Of particular interest is the important role of EGF in colony formation of smooth muscle cells (Fig. 4) despite its rather weak effect on stimulating mitosis of these cells in monolayer (Table I). An appropriate interpretation of these results will require further study.

Two differences between the effects of TGF-β on growth of smooth muscle and NRK cells are that colony formation of NRK cells is more efficient and requires less PDGF than does that of smooth muscle cells. Both of these differences may well be due to the nature of the NRK cells (line 49F) used in these studies; the cells were specifically selected for efficient growth in soft agar (Joseph De Larco, personal communication). For an unselected, early passage cell, the growth of explant cultures of bovine smooth muscle cells in soft agar is reasonably efficient: at least half of the seeded cells will divide in soft agar in response to optimum concentrations of growth factors, and ~15% of the cells will divide many times to form relatively large colonies (of >60 μm diam). The high amounts of PDGF needed to stimulate anchorage-independent growth of smooth muscle cells, in fact, complements other studies (1, 19, 22, 50) showing that cells of mesenchymal origin can have quantitatively different growth factor requirements for colony formation. When suspended in soft agar containing 10% calf

serum, colony formation of mesenchymal cells may require the addition of EGF (Rat-1 cells); TGF-β (AKR-2B cells); EGF and TGF-β (NRK cells); EGF, TGF-β, and insulin-like growth factor (BALB/c 3T3 cells); or EGF, TGF-β, and PDGF (smooth muscle cells; this report).

Studies by Seifert et al. (40) have shown that explant cultures of smooth muscle cells from pup, but not adult, rat aorta produce mitogenic concentrations of a PDGF-like peptide. Nilsson et al. (25) have reported that adult rat smooth muscle cells, isolated by collagenase digestion of aortic media, secrete a PDGF-like peptide upon their "modulation" to a mitotic phenotype. In contrast to these reports, the explant cultures of fetal bovine aortic smooth muscle cells used throughout this study did not appear to secrete large amounts of a PDGF-like peptide as determined by stimulation of mitosis with exogenous PDGF (Fig. 5) and induction of quiescence in subconfluent cultures with plasma-derived serum (Fig. 3A).

The results presented here raise the possibility that growth of vascular smooth muscle cells in response to platelet degranulation may not be due to PDGF alone but rather to an interaction of platelet-derived peptides that has the intrinsic ability to limit as well as stimulate mitosis. Interestingly, several lines of evidence suggest that the high concentrations of PDGF required to elicit the stimulatory effect of TGF-β in smooth muscle cell culture may be present during atherogenesis. First, as suggested by the response-to-injury hypothesis (35), platelet release may act as a point source for growth factors; the concentrations of TGF-β, PDGF, and the EGF-like peptide (4) may be high for aortic smooth muscle cells near sites of platelet release. Second, endothelial cells in culture produce PDGF-like peptides (10) and may supply medial smooth muscle cells with PDGF in vivo. Third, activated macrophages release PDGF in vitro, and these cells are an important component of the atherosclerotic aorta (43). Fourth, it is possible to prepare cultures of aortic smooth muscle cells that themselves produce a PDGF-like peptide (25, 51). Taken together with previous studies (11, 35, 36, 46, 52), the results presented here extend the interface of PDGF and TGF-β biology and suggest that both platelet peptides have potential roles in wound repair, transformation, and atherogenesis.

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