

# Transforming Growth Factor- $\beta$ Activity Is Potentiated by Heparin Via Dissociation of the Transforming Growth Factor- $\beta$ / $\alpha_2$ -Macroglobulin Inactive Complex

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**Abstract.** The control of smooth muscle cell (SMC) proliferation is determined by the combined actions of mitogens, such as platelet-derived growth factor, and the opposing action of growth inhibitory agents, such as heparin and transforming growth factor- $\beta$  (TGF- $\beta$ ). The present studies identify an interaction between heparin and TGF- $\beta$  in which heparin potentiates the biological action of TGF- $\beta$ . Using a neutralizing antibody to TGF- $\beta$ , we observed that the short term antiproliferative effect of heparin depended upon the presence of biologically active TGF- $\beta$ . This effect was observed in rat and bovine aortic SMC and in CCL64 cells, but not in human saphenous vein SMC. Binding studies demonstrated that the addition of heparin (100  $\mu$ g/ml) to medium containing 10% plasma-derived serum resulted in a 45% increase in the specific binding of  $^{125}$ I-TGF- $\beta$  to cells. Likewise, heparin induced a twofold increase in the growth inhibitory action of

TGF- $\beta$  at concentrations of TGF- $\beta$  near its apparent dissociation constant. Using  $^{125}$ I-labeled TGF- $\beta$ , we demonstrated that TGF- $\beta$  complexes with the plasma component  $\alpha_2$ -macroglobulin, but not with fibronectin. Heparin increases the electrophoretic mobility of TGF- $\beta$  apparently by freeing TGF- $\beta$  from its complex with  $\alpha_2$ -macroglobulin. Dextran sulfate, another highly charged antiproliferative molecule, but not chondroitin sulfate or dermatan sulfate, similarly modified TGF- $\beta$ 's mobility. Relatively high, antiproliferative concentrations of heparin (1–100  $\mu$ g/ml) were required to dissociate the TGF- $\beta$ / $\alpha_2$ -macroglobulin complex. Thus, it appears that the antiproliferative effect of heparin may be partially attributed to its ability to potentiate the biological activity of TGF- $\beta$  by dissociating it from  $\alpha_2$ -macroglobulin, which normally renders it inactive. We suggest that heparin-like agents may be important regulators of TGF- $\beta$ 's biological activity.

**A**THEROSCLEROSIS is characterized both by proliferative changes in the arterial cells and by biochemical alterations in the extracellular matrix that lead to local occlusive and thrombotic complications. The intimal invasion and proliferation of vascular smooth muscle cells (SMCs)<sup>1</sup> may be a key step in the pathogenesis of atherosclerosis (51). After arterial injury, humans and a variety of other species develop a neointimal vascular lesion composed partially of cells expressing SMC antigens (50, 62). In experimental animals, formation of arteriosclerotic lesions is inhibited by heparin pretreatment (8, 17). This effect of heparin correlates with its ability to inhibit arterial SMC growth in vitro (4, 12, 21, 41, 47), and is distinct from its anticoagulant action (17, 33). The proliferation of other cell types such as fibroblasts, skeletal muscle cells, and tumor cell lines is also inhibited by heparin (10, 24, 28, 33).

The antiproliferative effect of heparin may be of physiolog-

ical significance because endothelial cells and postconfluent SMCs produce heparan sulfate proteoglycans that act similarly to heparin (16, 28). Likewise, highly sulfated polysaccharides such as dextran sulfate and mactin can also have antiproliferative effects (33). Other cell-derived glycosaminoglycans, such as chondroitin sulfate and dermatan sulfate, do not exert antiproliferative effects. We have previously reported that heparin corrects the excessive proliferation of SMC derived from aged rats (38). We subsequently observed, as did others (1), that transforming growth factor type beta (TGF- $\beta$ ) was also a potent inhibitor of SMC monolayer growth in vitro. The inhibition induced by TGF- $\beta$ , however, was observed at almost a 1,000-fold lower molar concentration than has been reported for heparin. To study the mechanism of heparin's antiproliferative effect, we tested the hypothesis that heparin inhibited SMC proliferation by stimulation of TGF- $\beta$  activity. Even though TGF- $\beta$  has not been regarded as a heparin-binding growth factor, our findings demonstrate a significant and specific biological interaction between heparin and TGF- $\beta$  that could contribute to the antiproliferative effect of heparin on vascular SMC as well as other cell types.

1. *Abbreviations used in this paper:*  $\alpha_2$ -M,  $\alpha_2$ -macroglobulin; CPC, cetylpyridium chloride; ITS, insulin-transferrin-selenium; PDS, plasma-derived bovine serum; SMC, smooth muscle cell; TGF- $\beta$ , transforming growth factor beta.

## Materials and Methods

### Reagents

Human TGF- $\beta$  (type 1) and PDGF (>99% pure) were purchased from R & D Systems (Minneapolis, MN). FBS was from Flow Laboratories, Inc. (McLean, VA) and plasma-derived bovine serum (PDS) was from Biomedical Technologies, Inc. (Cambridge, MA). Tritiated thymidine (1 mCi/ml; New England Nuclear, Boston, MA) was used for proliferation assays.  $\alpha_2$ -Macroglobulin ( $\alpha_2$ -M) was kindly provided by Dr. Peter Harpel (Cornell University Medical College, New York). Fibronectin was isolated from human serum by published techniques (14). Sodium heparin, dermatan sulfate, and dextran sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Chondroitin-6-sulfate was acquired from the Seikagaku Kogyo Co. (Tokyo, Japan) through Miles Laboratories (Naperville, IL). Heparin of low anticoagulant activity (RD7; 7 U/mg) was kindly provided by the Hepar Corp. (Chicago, IL). A highly charged heparin species was recovered from commercially available heparin (Sigma Chemical Co.) by precipitation with cationic cetylpyridium chloride (CPC). The heparin-CPC complex was partially dissolved with 1.6 M NaCl/0.05 M CPC. Complexes resistant to 1.6 M NaCl were recovered and dissolved in 2 M NaCl/absolute ethanol (100:15, vol/vol). This heparin fraction (Hep<sub>1,6</sub>) was purified by several cycles of ethanol precipitation. Unless otherwise specified, heparin used in these experiments is commercially available Sigma heparin.

### Cell Culture

Aortic SMCs were obtained from several sources. Bovine aortic SMCs isolated from explants of fresh bovine aortic media were kindly provided by Drs. David Hajjar and Andrew Nicholson (Cornell University Medical College). Rat aortic SMCs were isolated by explant from male Fisher 344 rats that had undergone balloon catheter-induced aortic deendothelialization 2 wk previously (40, 58). Human saphenous vein SMCs were isolated from explanted medial segments of vein segments remaining after coronary artery bypass and obtained in accordance with Institutional Review Board approved protocols. Cultured cells were confirmed as SMCs by positive staining for the smooth muscle-specific isoform of actin as recognized by the monoclonal antibody HHH35 (kindly provided by Dr. Allen Gown, University of Washington, Seattle, WA). Cultures of SMCs were used in the second to sixth subpassage. Mink lung epithelial cells (CCL64) were obtained from the American Type Culture Collection (Rockville, MD) in the 60th passage and used in passages 62–68. All cell cultures were maintained in medium 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS and 50  $\mu$ g/ml gentamicin sulfate (Gibco Laboratories). Subpassage was achieved with trypsin/EDTA (Flow Laboratories) and a 3:1 split ratio.

### Proliferation Assays

The inhibitory effect of heparin on SMC proliferation, and the contribution of TGF- $\beta$  to that activity, was evaluated by a short term assay of tritiated thymidine incorporation into DNA. This design was used to minimize the possible contributions of any cell-derived inhibitors. SMCs were plated in normal culture medium at 10,000 cells/well in 96-well microtiter plates. After 48 h, the cells were washed once with plain medium 199 and then exposed to the test medium for 14 h before addition of tritiated thymidine (1  $\mu$ Ci/well) for 4 h before harvest. Incorporation of radiolabeled thymidine into DNA was measured by published methods (38). Reported values reflect the average of six replicate wells per experiment. Inhibition of TGF- $\beta$  activity by specific antibody was achieved by preincubating the test medium for 1 h with a 1:10 dilution of a 1 mg/ml solution of rabbit anti-human TGF- $\beta$  (R & D Systems) known to neutralize the biological activity of  $\sim$ 2 ng/ml TGF- $\beta$  (25). Control groups were incubated with an identical dilution of nonimmune rabbit IgG.

### Iodination of TGF- $\beta$

TGF- $\beta$  was iodinated by minor modifications of the chloramine-T method of Ruff and Rizzino (52). Radiolabeled protein normally exhibited a specific activity of  $2.5 \times 10^5$  cpm/ng protein and migrated as a single DTT-reducible band on SDS-PAGE. Comparison of radiolabeled and unlabeled protein in a growth inhibition assay using CCL64 fibroblasts indicated equivalent biological activities. Inhibition of growth in these studies was determined either by cell counts on a ZBI counter (Coulter Electronics Inc., Hialeah, FL) or by determining DNA content per well by a fluorometric assay in mi-

croter wells, using a previously published method developed by the authors (37).

### Binding Studies

Binding studies were performed essentially as described by Ruff and Rizzino (52). CCL64 cells were plated in normal growth medium in 24-well Costar (Cambridge, MA) plates at a density of 50,000 cells per well for 48 h. The cells were then washed twice with binding buffer (medium 199 plus 0.1% BSA) and iodinated TGF- $\beta$  was applied to the cells in medium 199 plus 10% PDS. All binding studies were performed after preequilibration of the cells and medium in a 4°C cold room and during gentle agitation on a rotary shaker. Nonspecific binding was assessed by adding 100 ng/ml (>100-fold excess) unlabeled crude TGF- $\beta$  (20% pure; R & D Systems) at the time the iodinated ligand was added. 2 h later, the cells were washed three times with ice cold binding medium and bound radioactivity was extracted by treating the cells with lysing medium (PBS plus 0.1% Triton X-100 and 10% glycerol). Previous studies have established that this method extracts a large percentage of the cell-bound ligand without releasing the nonspecifically bound counts (52).

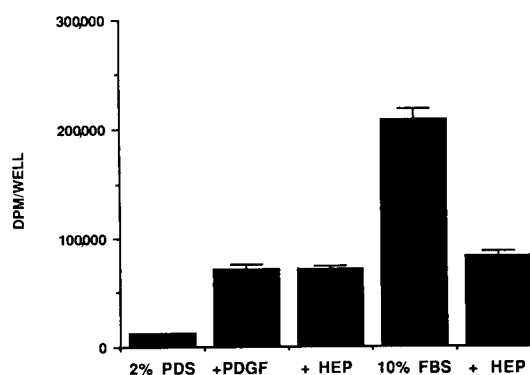
### Agarose Gel Electrophoresis

The effect of heparin on the electrophoretic mobility of <sup>125</sup>I-TGF- $\beta$  was analyzed by electrophoresis in uncharged, 1% composite agarose-galactomannan gels (Isogel; FMC Bioproducts Corp., Rockland, ME). Gels prepared with 50 mM Tris-acetate buffer (pH 7.0) were run at 150 V/20 mA for  $\sim$ 4 h with cooling. After electrophoresis, sample wells were filled with molten agarose and allowed to cool to prevent the loss of unmigrated TGF- $\beta$ . Proteins were fixed in 10% trichloroacetic acid for 1 h and then washed free of acid and buffer overnight in water. After drying, gels were stained with 0.1% Coomassie brilliant blue R in water/ethanol/acetic acid (65:25:10, vol/vol/vol), then destained, and exposed overnight to Kodak XAR film (Eastman Kodak Co., Rochester, NY) above a Cronex Lightning Plus intensifying screen.

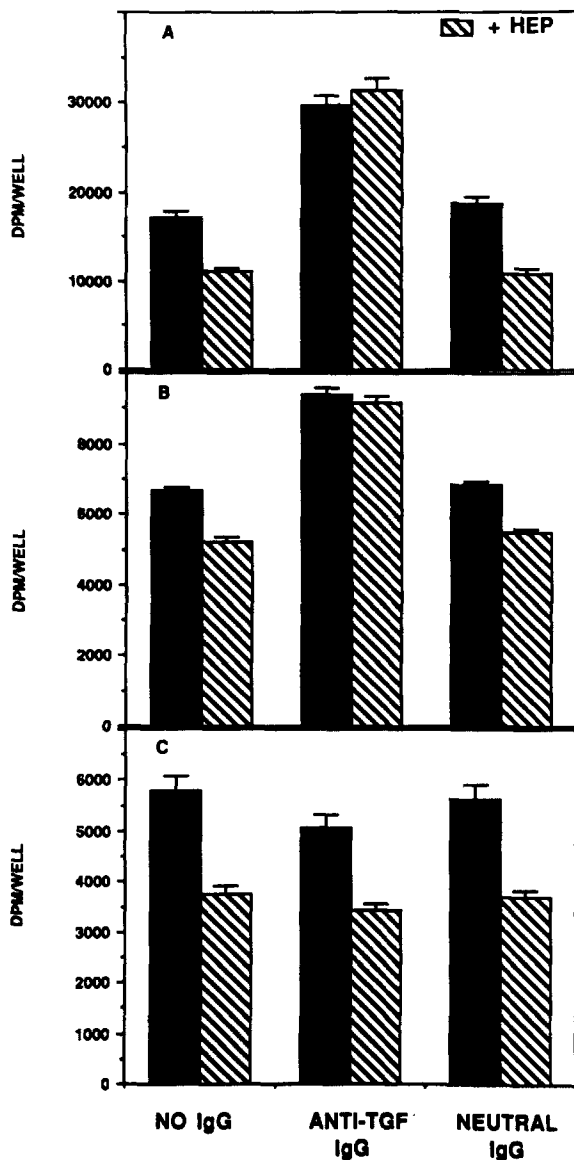
## Results

### Serum Dependence of the Antiproliferative Effect of Heparin

The antiproliferative effect of heparin on SMCs has been observed under culture conditions that included serum (48). We had previously observed that the proliferation of SMCs derived from young (3–4-mo) rats cultured in 2% PDS and stimulated with PDGF was not inhibited by heparin (38). To



**Figure 1.** Serum dependence of heparin's antiproliferative effect. Rat aortic SMC were plated in 10% FBS 48 h before exposure to the following culture conditions. 18 h after exposure to 2% PDS, 2% PDS + 10 ng/ml PDGF, PDGF + heparin (100  $\mu$ g/ml), 10% FBS, or 10% FBS + heparin, the cells were pulsed with tritiated thymidine (1  $\mu$ Ci/well) for 2 h. The data points are the mean  $\pm$  SEM of six replicate wells.



**Figure 2.** Reversibility of heparin-induced inhibition by antibody to TGF- $\beta$ . (A) Bovine aortic SMC, (B) rat aortic SMC, and (C) human saphenous vein SMC cells were plated in normal culture medium 48 h before the experiment. The medium was then changed to medium plus 5% FBS with (hatched bars) or without (solid bars) addition of heparin (100  $\mu$ g/ml) for 18 h before exposure to a 4-h pulse of tritiated thymidine. In the specified groups, antibody to TGF- $\beta$  or nonimmune rabbit IgG was added at 100  $\mu$ g/ml to medium plus 5% FBS 1 h previous to adding this to cells. Data points are the mean  $\pm$  SEM of six replicate wells.

compare the inhibitory effect of heparin on the proliferation of cells stimulated with serum to the effect of heparin on cells stimulated with PDGF, rat SMCs were changed from normal culture medium to either 2% PDS plus PDGF (10 ng/ml) and insulin-transferrin-selenium (ITS; Sigma Chemical Co.) or to 10% FBS plus ITS. Parallel groups of cells in both media were also treated with heparin (100  $\mu$ g/ml) for 18 h before a 2-h exposure to tritiated thymidine. As Fig. 1 demonstrates, the PDGF-stimulated rate of DNA synthesis in media containing 2% PDS is not altered by heparin. Whole blood serum-stimulated (10% FBS) DNA synthesis, however, is

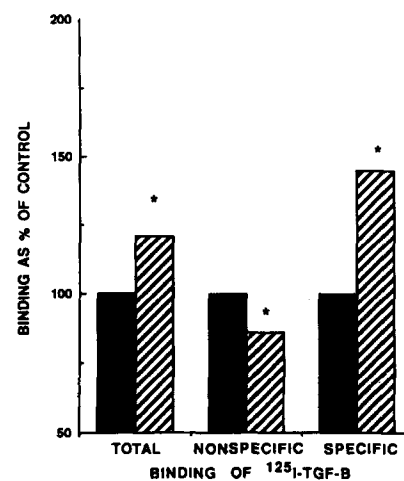
strongly suppressed by heparin to approximately the level observed with PDGF stimulation. The initial levels of stimulated DNA synthesis differ between the PDGF- and serum-stimulated groups. Nonetheless, the PDGF-treated cells show stimulated DNA synthesis which was not inhibited by heparin to the level of the 2% PDS control.

### *The Effects of Antibody to TGF- $\beta$ on the Growth of SMCs*

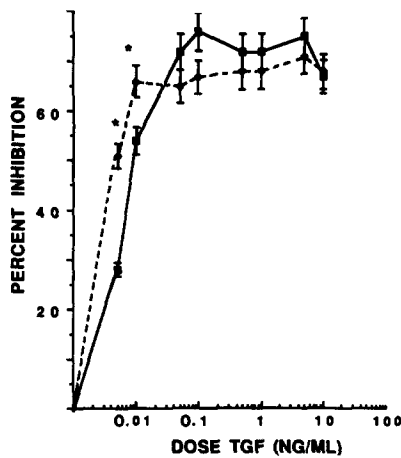
In parallel studies using rat SMC cultures grown in 2% PDS, it was observed that a neutralizing titer of antibody to TGF- $\beta$  (100  $\mu$ g/ml) caused a 36% increase in the rate of DNA synthesis above control (control = 4,576  $\pm$  356 dpm/well; anti-TGF- $\beta$  = 6,206  $\pm$  482 dpm/well; mean  $\pm$  SEM,  $n$  = 10). In the complete absence of PDS or FBS in the culture media, anti-TGF- $\beta$  essentially doubled the rate of thymidine incorporation (medium 199 + ITS = 12,367.4  $\pm$  902.3 dpm/well vs. antibody-treated wells = 23,265.2  $\pm$  2,183.9 dpm/well,  $n$  = 5). Nonimmune IgG has no effect on the rate of thymidine incorporation in these cells (Fig. 2). Because this antibody-stimulated DNA synthesis was observed in cells that were presumably devoid of exogenous TGF- $\beta$ , it suggests that these rat SMCs were producing TGF- $\beta$  which was inhibiting their own growth in an autocrine fashion. The contribution of serum- and cell-derived TGF- $\beta$  to heparin's antiproliferative effect was examined in the following study.

### *Reversal of Heparin Inhibition by Antibody to TGF- $\beta$*

The apparent dependence of heparin's antiproliferative effect on a serum component suggests the possible involvement of a platelet-derived substance, such as TGF- $\beta$ , that is present in whole blood serum but not in plasma-derived serum. To



**Figure 3.** Effects of heparin on the binding of  $^{125}$ I-TGF- $\beta$ . CCL64 cells were plated in normal culture medium 48 h before the experiment at a density of 25,000 cells/well of a 24-well plate. Binding of 0.1 ng/well (25,000 cpm)  $^{125}$ I-TGF- $\beta$  was assessed in the presence (nonspecific) or absence (total) of a >100-fold excess of unlabeled TGF- $\beta$ . Data points represent the mean of quadruplicate determinations in each of three replicate experiments. The cpm's corresponding to the 100% value for each measure of binding are total = 621.2  $\pm$  110 (SEM), nonspecific = 295.8  $\pm$  95, and specific = 325.3  $\pm$  39. Asterisks indicate control (solid bars) and heparin (hatched bars) groups are significantly different,  $P$  < 0.05.



**Figure 4.** Effects of heparin on the antiproliferative action of TGF- $\beta$ . CCL64 cells were plated at 10,000 cells per microtiter well in normal culture medium 48 h before use. The cells were then changed to medium plus 5% PDS and increasing doses of TGF- $\beta$  with (dashed line) or without (solid line) 100  $\mu$ g/ml heparin for 18 h before exposure to a 4-h pulse of tritiated thymidine. Each point represents the mean  $\pm$  SEM,  $n = 6$ ; asterisks indicate that control and heparin groups are significantly different,  $P < 0.01$ .

test the hypothesis that heparin potentiates TGF- $\beta$  activity present in serum, or produced by the SMCs themselves, we examined the ability of heparin to inhibit proliferation in the presence or absence of an antibody known to neutralize TGF- $\beta$ . As demonstrated in Fig. 2, heparin was an effective growth inhibitor for bovine SMCs (Fig. 2A), rat SMCs (Fig. 2B), and human saphenous vein SMCs (Fig. 2C). In both the bovine and rat arterial SMC cultures, however, heparin did not inhibit DNA synthesis when the culture was pretreated with antibody to TGF- $\beta$ . Similar results were obtained even when CCL64 were used as target cells (data not shown). Furthermore, in the presence of anti-TGF- $\beta$  DNA synthesis by SMCs was stimulated, suggesting that the concentration of TGF- $\beta$  present in medium containing 5% FBS was inhibitory to proliferation. Heparin also inhibited the growth of saphenous vein SMCs, but in these cells the inhibitory effect persisted in the presence of antibody to TGF- $\beta$ , suggesting either incomplete neutralization of the TGF- $\beta$ , or a second pathway for the inhibitory effect of heparin. However, the proliferation of the saphenous vein SMCs was not inhibited by TGF- $\beta$  as determined not only by the absence of an antibody effect, but also by treating replicate wells directly with TGF- $\beta$  (data not shown). Yet, in three of the four cell strains tested, the inhibitory action of heparin was blocked by antibody to TGF- $\beta$ .

#### Effects of Heparin on the Binding of TGF- $\beta$

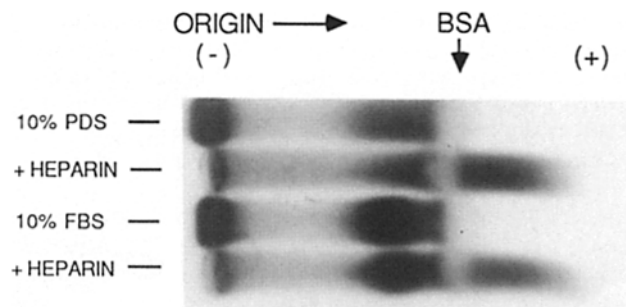
Having demonstrated that heparin loses its short term antiproliferative effect in the absence of biologically active TGF- $\beta$ , we addressed the possibility that heparin potentiates the activity of TGF- $\beta$  by increasing the specific binding of TGF- $\beta$  to cells. This possibility was examined by determining if heparin altered specific binding of radiolabeled TGF- $\beta$  to cells under conditions similar to those of the growth inhibition assay. We chose CCL64 cells for this assay because of their sensitivity to the antiproliferative action of TGF- $\beta$

and their ease of handling. Scatchard analysis of the binding of radiolabeled TGF- $\beta$  to CCL64 cells indicated the presence of a very high affinity, low capacity binding site with an apparent  $K_d$  of 8.3 pM (not shown). We chose a subsaturating concentration of TGF- $\beta$  (9.8 pM, 25,000 cpm/well), near the apparent dissociation constant, to examine the effects of heparin on TGF- $\beta$  binding.

To approximate cell culture conditions, the binding studies were conducted in the presence of 10% PDS with or without the addition of heparin (100  $\mu$ g/ml). Whole blood serum could not be used in these studies because of the confounding effects of unlabeled serum TGF- $\beta$ . Preliminary experiments (not shown) indicated that the presence of 10% PDS or 100  $\mu$ g/ml of  $\alpha_2$ -M reduced both total and specific binding of  $^{125}$ I-TGF- $\beta$  to these cells, consistent with previous reports (42). Heparin partially reversed the effect of PDS, increasing the specific binding of TGF- $\beta$  to the cell monolayer (Fig. 3). Addition of heparin to the binding medium increased specific binding in two ways: (a) reducing nonspecific binding (binding in the presence of a >100-fold excess of cold competitor) to 86% of the level observed in untreated wells ( $t[13] = 3.3$ ,  $P = 0.006$ ); and (b) increasing the total binding of TGF- $\beta$  to 21% above untreated wells ( $t[13] = 2.74$ ,  $P = 0.017$ ). The net effect of these two factors is to increase specific binding of TGF- $\beta$  to cells by 46% above that observed in the absence of heparin ( $t[13] = 3.3$ ,  $P = 0.006$ ). This heparin-stimulated binding was not observed in two studies (not shown) in which only 1% BSA was used as the binding medium, but was observed when 10% PDS was used. Heparin-stimulated binding is observed when binding is performed either at 4°C, as described above, or at 37°C (data not shown) which, combined with the previous evidence, implies that heparin does not affect the levels of the TGF- $\beta$  receptor(s). However, the presence of a certain type of receptor may be required because saphenous vein SMCs did not show heparin-sensitive binding of TGF- $\beta$ , an effect consistent with our finding that anti-TGF- $\beta$  does not reverse heparin's inhibitory effect on these cells.

#### Effect of Heparin on the Biological Action of TGF- $\beta$

The fact that heparin potentiated the specific binding of TGF-



**Figure 5.** Effect of heparin on the electrophoretic mobility of TGF- $\beta$  in serum or plasma.  $^{125}$ I-TGF- $\beta$  in 1% carrier BSA was mixed with medium 199 and 10% PDS or 10% whole-blood FBS in the presence or absence of 100  $\mu$ g/ml heparin and electrophoresed in a 1% agarose-galactomannan gel under nonreducing and nonreducing conditions. The gel was fixed and then autoradiographically exposed overnight. The arrow indicates the migration of the BSA vehicle as determined by Coomassie staining.

$\beta$  suggested that heparin might also potentiate the biological action of TGF- $\beta$ . To assess this possibility, the antiproliferative effect of TGF- $\beta$  was measured in the presence or absence of heparin (100  $\mu$ g/ml). In cells plated in 10% FBS and then changed to 5% PDS, heparin alone had a significant inhibitory effect on the baseline rate of thymidine incorporation (no heparin or TGF- $\beta$  [control] = 7,727  $\pm$  447 dpm/well; with heparin = 3,581  $\pm$  443 dpm/well; mean  $\pm$  SEM,  $n$  = 6). To evaluate the heparin/TGF- $\beta$  interaction, we expressed the effect of each concentration of TGF- $\beta$  as a percent inhibition relative to its own control or the heparin baseline value just mentioned. As Fig. 4 demonstrates, heparin increased the inhibitory effect of the lowest tested concentrations of TGF- $\beta$  without substantially altering the maximal inhibitory effect. The inhibitory effect of 50 pg/ml TGF- $\beta$ , for instance, was increased twofold in the presence of heparin ( $P$  < 0.01). Thus, at low concentrations of TGF- $\beta$ , heparin potentiates its antiproliferative effect.

### Effect of Heparin on the TGF- $\beta$ / $\alpha_2$ -M Complex

The results of the previous experiments suggested that heparin exerts an antiproliferative effect that correlates with its ability to potentiate the binding and biological action of TGF- $\beta$ . Platelet-derived or cell-secreted TGF- $\beta$  that is not receptor bound is rapidly complexed with  $\alpha_2$ -M, greatly reducing its biological activity (42). Other reports suggest that TGF- $\beta$  also complexes with fibronectin in plasma (15). We examined the possibility that heparin binds to the inactive TGF- $\beta$ / $\alpha_2$ -M complex in plasma or serum and induces a conformational change or dissociation that exposes the active site of TGF- $\beta$  to cell surface receptors.

We examined this hypothesis by adding  $^{125}$ I-TGF- $\beta$  to plasma or serum and determining the ability of heparin to modify the electrophoretic mobility of the complex. Fig. 5 represents the autoradiograph of radiolabeled TGF- $\beta$  complexed to plasma or serum components and analyzed by non-denaturing 1% agarose gel electrophoresis. Under these conditions, TGF- $\beta$  that is either free or is complexed to an uncharged protein does not enter the gel, due to its weak cationic nature (23). Migration of TGF- $\beta$  into the gel is dependent upon formation of a complex with a charged protein and is largely independent of the molecular weight of the complex. Complexes formed in the presence of either FBS or PDS appear to be modified by the addition of heparin at antiproliferative doses as indicated by the increased cathodic migration of the radiolabeled TGF- $\beta$  (Fig. 5). This suggests that heparin binds directly to TGF- $\beta$  or to a protein complex containing TGF- $\beta$ .

As shown in Fig. 6, the addition of heparin to  $^{125}$ I-TGF- $\beta$  increases the migration of TGF- $\beta$  into the gel. This strongly suggests that there is a direct interaction between TGF- $\beta$  and heparin. However, the possibility remains that TGF- $\beta$  is complexed to a trace contaminant in the albumin carrier which is affected by heparin. Also, as shown in Fig. 6, TGF- $\beta$  forms a complex with purified  $\alpha_2$ -M. The addition of heparin to TGF- $\beta$ / $\alpha_2$ -M complexes resulted in the enhanced migration of a large fraction of the radiolabeled TGF- $\beta$  that was previously comigrating with  $\alpha_2$ -M. When TGF- $\beta$  was added to fibronectin, we did not observe the same degree of complex formation that we observed with  $\alpha_2$ -M. The addition of heparin, however, created the same highly anionic band that was observed when heparin was added to TGF- $\beta$  alone or in

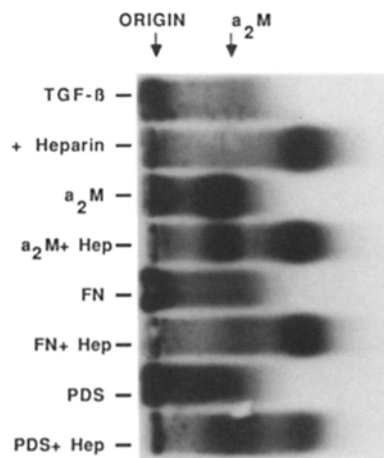


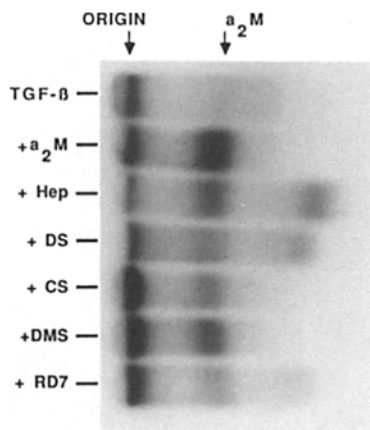
Figure 6. Effect of heparin on complex formation of  $^{125}$ I-TGF- $\beta$  with plasma proteins or purified  $\alpha_2$ -M. Agarose gel electrophoresis as described for Fig. 5.  $^{125}$ I-TGF- $\beta$  in 1% BSA carrier was mixed with medium 199 and from top to bottom: 100  $\mu$ g/ml heparin, 100  $\mu$ g/ml  $\alpha_2$ -M( $\alpha_2$ M),  $\alpha_2$ -M + heparin ( $\alpha_2$ M + Hep), 100  $\mu$ g/ml fibronectin (FN), fibronectin + heparin (FN + Hep), 10% PDS, or 10% PDS + heparin (PDS + Hep). Arrow indicates migration of purified  $\alpha_2$ -M as determined by Coomassie staining.

combination with  $\alpha_2$ -M. Densitometric analysis of the Coomassie-stained gel indicated that heparin did not shift the  $\alpha_2$ -M band. In contrast, fibronectin's mobility was significantly enhanced by heparin, consistent with previous reports (14). However, in the presence of heparin the radiolabeled TGF- $\beta$  did not comigrate with fibronectin/heparin complexes. These data suggest that heparin binds to TGF- $\beta$  and thereby dissociates the TGF- $\beta$ / $\alpha_2$ -M complex.

As shown in Fig. 7, the dissociation of the TGF- $\beta$ / $\alpha_2$ -M complex was observed with heparin and dextran sulfate, both of which are antiproliferative (33). RD7, a heparin with low anticoagulant activity but some antiproliferative activity (data not shown), weakly dissociated the TGF- $\beta$ / $\alpha_2$ -M complex. Chondroitin sulfate and dermatan sulfate, which are not antiproliferative (17), do not modify TGF- $\beta$ / $\alpha_2$ -macroglobulin complexes (Fig. 7). To minimize the possible contribution of any trace contaminants in commercial heparin preparations, we used a highly purified heparin to determine the dose-response characteristics of this interaction. As demonstrated in Fig. 8, the dissociation of the TGF- $\beta$ / $\alpha_2$ -M complex by heparin requires an excess of highly purified heparin (HEP<sub>1.6</sub>) as determined by increasing the concentration of heparin (1 ng/ml to 100  $\mu$ g/ml) relative to fixed concentrations of  $^{125}$ I-TGF- $\beta$  (20 ng/ml) and  $\alpha_2$ -M (100  $\mu$ g/ml). As the concentration of heparin is increased, the electrophoretic mobility of  $^{125}$ I-TGF- $\beta$  shows a marked cathodic shift, while the position of the  $\alpha_2$ -M on the Coomassie-stained gel was unchanged. Thus, the concentrations of heparin sufficient to free  $^{125}$ I-TGF- $\beta$  from  $\alpha_2$ -M are quite similar to the antiproliferative concentrations of heparin reported by several laboratories (10, 12, 22, 24, 38, 47).

### Discussion

TGF- $\beta$  is a widely expressed and pluripotent growth factor. Platelets contain, and upon activation release, a high molecular weight, latent form of TGF- $\beta$  (67) in quantities sufficient



**Figure 7.** Specificity of heparin for the TGF- $\beta$ / $\alpha_2$ -M complex. Agarose gel electrophoresis and autoradiography performed as in Fig. 5. Purified  $\alpha_2$ -M ( $\alpha_2$ M) (100  $\mu$ g/ml) and  $^{125}$ I-TGF- $\beta$  were mixed with or without 100  $\mu$ g/ml of heparin (Hep), dextran sulfate (DS), chondroitin-6-sulfate (CS), dermatan sulfate (DMS), or RD7, a heparin with antiproliferative but low anticoagulant activity. All polysaccharides were used at 100  $\mu$ g/ml.

to generate approximately equimolar concentrations of PDGF and TGF- $\beta$  in serum (see reference 57 for review). A variety of cell types, including fibroblasts (25, 29), macrophages (45), and tumor cell lines (7, 56) produce TGF- $\beta$ . Specific, high affinity receptors for TGF- $\beta$  have also been observed in almost all cell types examined (11, 26, 35, 36, 49, 54, 66). The fact that cells can produce levels of TGF- $\beta$  to which the same cells will respond classifies TGF- $\beta$  as a potentially important autocrine growth regulator (25). The biological responses to TGF- $\beta$  binding are quite varied: modulation of cellular phenotype (35); and increased synthesis of extracellular collagen (43, 46, 64, 68), fibronectin (43, 64, 68), thrombospondin (43), plasminogen activator inhibitor (68), and glycosaminoglycans (3, 5, 13, 46) are observed in several cell types including SMC. Increased monocyte chemotaxis (65), growth factor production (32, 65), and autoinduction of TGF- $\beta$ 1 expression (63) have all been observed after TGF- $\beta$  exposure.

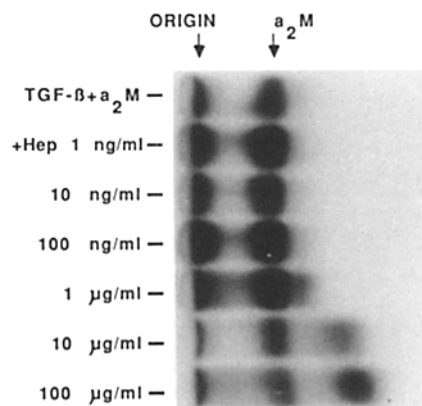
TGF- $\beta$  is a potent growth inhibitor for cultured endothelial cells (2, 19, 59) and B lymphocytes (55). SMC and fibroblasts are growth stimulated or inhibited depending on the particular culture conditions (20, 22, 27, 35). TGF- $\beta$  is produced in an acid-activatable latent form by fibroblasts (30, 31); and in platelets, TGF- $\beta$  exists in a latent complex with a putative precursor polypeptide (39, 67). While the precise activation mechanism is uncertain, it seems likely that the complex is proteolytically activated by a plasmin-like enzyme to form a 26-kD biologically active molecule (34). Active TGF- $\beta$  is rapidly bound to  $\alpha_2$ -M (23), presumably serving as a clearance mechanism (9). Thus, TGF- $\beta$  may exist in two distinct, biologically inactive forms: the original latent complex with its precursor and a second clearance complex with  $\alpha_2$ -M.

Although TGF- $\beta$  has not previously been considered a heparin-binding growth factor, our experiments indicate that heparin may have important biochemical and physiological interactions with this potent growth and metabolic regulator. Because heparin is known to interact with acidic fibroblast growth factor (aFGF, also known as heparin-binding growth

factor-1) to potentiate its mitogenic effect on endothelial cells (61), an analogous interaction between heparin and TGF- $\beta$  is possible. While it has recently been shown that TGF- $\beta$  binds to heparin-Sepharose with greater affinity than does PDGF (25, 45), the physiological significance for this interaction was not clear. Our studies strongly suggest that at least one of the growth inhibitory actions of heparin is to potentiate the receptor binding and antiproliferative effect of TGF- $\beta$  via dissociation of the  $\alpha_2$ -M complex. The evidence supporting this interaction is derived from several observations.

First, heparin is a much less effective inhibitor of cell growth in the absence of serum derived from whole blood, which contains both PDGF and TGF- $\beta$  complexed to protein macromolecules. Initially this observation seemed to conflict with the findings of Reilly and co-workers (47) who reported that heparin did inhibit PDGF-stimulated growth. However, closer examination of the reported methods indicated that 5% calf serum, and thus TGF- $\beta$ , was present in the PDGF-supplemented medium. Heparin has been reported to inhibit SMC proliferation in culture conditions using serum depleted of PDGF by heparin-Sepharose chromatography (4, 47). Although these reports have been interpreted as evidence that heparin does not interact with a serum factor to cause growth inhibition, our results indicate that an interaction between  $\alpha_2$ -M, which is not removed from serum by heparin-Sepharose, and TGF- $\beta$ , which is only partially removed by heparin-Sepharose (25) and could be supplied by the cultured cells, is still a plausible mechanism for heparin's antiproliferative effect in this particular setting.

The contribution of TGF- $\beta$  to heparin's antiproliferative effect is further supported by the fact that in the presence of a neutralizing antibody to TGF- $\beta$ , heparin did not inhibit SMC proliferation. That TGF- $\beta$  present in serum inhibits SMC growth can be strongly inferred from the substantial increase in DNA synthesis in cells treated with antibody to TGF- $\beta$ . This "disinhibition" is attributable to the neutralization of TGF- $\beta$  present in serum. A similar stimulatory effect of anti-TGF- $\beta$  is also observed in serum-free cultures of SMC, suggesting that SMCs produce TGF- $\beta$  that regulates their own growth. This has not been previously reported for



**Figure 8.** Effect of increasing heparin concentrations on complex formation. Agarose gel electrophoresis as in Figs. 5-7 showing the requirement of a >50-fold molar excess of purified heparin (Hep<sub>1,6</sub>) to TGF- $\beta$  (20 ng/ml) is necessary to cause dissociation of the  $\alpha_2$ -M/ $^{125}$ I-TGF- $\beta$  complex.  $\alpha_2$ -M was present in all lanes at a concentration of 100  $\mu$ g/ml.

SMCs though it is known that other cell types produce TGF- $\beta$  in an autocrine fashion (25). The failure of heparin to inhibit DNA synthesis in the absence of functional TGF- $\beta$  implies that heparin might facilitate the interaction of TGF- $\beta$  with its receptor.

To test that possibility, it was necessary to examine the cell surface binding of TGF- $\beta$  in the presence and absence of heparin. At concentrations of TGF- $\beta$  near its apparent dissociation constant, heparin increased the specific binding of TGF- $\beta$  by  $\sim 45\%$  above untreated control wells. Part of this effect was to reduce nonspecific binding, suggesting that part of TGF- $\beta$ 's binding to cells is via a heparin-sensitive site. This is consistent with the recent report that the high molecular weight receptor for TGF- $\beta$  is a glycosaminoglycan and that heparinase digestion reduces TGF- $\beta$  binding to the cells by  $\sim 20\%$  (54). Consistent with the increased binding of TGF- $\beta$ , heparin also potentiated the growth inhibitory action of very low concentrations of TGF- $\beta$ . Taken together, it seems reasonable to hypothesize that heparin increases the availability of TGF- $\beta$  to its receptor, and thereby potentiates the antiproliferative effect of TGF- $\beta$  on these cell types.

One mechanism by which heparin could influence the binding and action of TGF- $\beta$  would be for heparin to bind to the TGF- $\beta$  complex that is formed in serum or plasma and render free TGF- $\beta$ . It has been established that TGF- $\beta$  binds to  $\alpha_2$ -M in plasma (23) and is less biologically active when so bound (42). Because TGF- $\beta$ , but not  $\alpha_2$ -M, binds heparin, we were not surprised to find that the electrophoretic mobility of TGF- $\beta$  bound to plasma proteins was substantially altered by the presence of highly electronegative heparin. This heparin/TGF- $\beta$  interaction exhibited glycosaminoglycan specificity and dose-response characteristics that parallel those described for heparin's antiproliferative effect. Based on these results, we propose that heparin frees TGF- $\beta$  from its binding site to  $\alpha_2$ -M and forms a stable, electronegative complex that is receptor competent. The large molar excess of heparin necessary to achieve this dissociation suggests that either a small subtype of heparin is effective or that heparin has a lower affinity than  $\alpha_2$ -M for TGF- $\beta$ .

An interaction between TGF- $\beta$  and heparin would have broad physiological implications because both TGF- $\beta$  and heparin-like glycosaminoglycans are produced in substantial quantities by a variety of cell types. Endothelial cells, for instance, produce heparan sulfate species (69), as well as relatively high levels of latent TGF- $\beta$ , implying that the combined effect of these factors may help to maintain cell cycle quiescence in the underlying SMC. Furthermore, because TGF- $\beta$  is known to increase the synthesis of matrix proteoglycans, there is the potential for feedforward/feedback mechanisms to regulate the extracellular activity of TGF- $\beta$  during wound healing, angiogenesis, and potentially atherogenesis. It is interesting to speculate that a TGF- $\beta$ /heparin interaction may also be involved in the regulation of bone integrity. Long term heparin therapy is often accompanied by osteoporosis (53), and TGF- $\beta$  is already well recognized as having important functions in the proliferation and metabolism of osteoclasts and osteoblasts (6, 44, 60). In conclusion, our results suggest the possibility that the interaction of heparin, or possibly heparan sulfate proteoglycans, and TGF- $\beta$  has important regulatory functions in both normal and pathological conditions of the vasculature. Further investigation of

this interaction will allow a better understanding of the factors that govern the balance of growth stimulatory and growth inhibitory signals acting on the mammalian cell.

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