Journal of Cellular Biochemistry 47:31–42 (1991)

Platelet Factor 4 Selectively Inhibits Binding of TGF-β_1 to the Type I TGF-β_1 Receptor

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Abstract A low molecular weight inhibitor of TGF-β_1 binding was detected in partially purified human platelet extracts by using Hep 3B hepatoma cells in the binding assays. The inhibitory protein was purified to homogeneity and was identified as platelet factor 4 on the basis of its amino acid sequence. TGF-β_1 binding to Hep 3B cells was almost completely inhibited by 100 nM concentrations of platelet factor 4, but TGF-β_1 binding to NRK 49F fibroblasts was inhibited only slightly. Affinity cross-linking experiments revealed that these differences in the inhibition of TGF-β_1 binding by platelet factor 4 were due to differences in the complements of TGF-β_1 binding proteins present on these two cell types. In Hep 3B cells the majority of bound TGF-β_1 was cross-linked to a complex which had an apparent molecular weight of 70 kDa. TGF-β_1 binding to this protein was the most sensitive to inhibition by platelet factor 4. Based on its size and TGF-β_1 binding properties, we believe this protein is the type I TGF-β_1 receptor. Hep 3B cells also had a high-affinity TGF-β_1 binding protein which appeared as an 80 kDa complex, and which we believe to be the type II TGF-β_1 receptor. TGF-β_1 binding to this protein was not inhibited by platelet factor 4. TGF-β_1 was also cross-linked to complexes of higher molecular weights in Hep 3B cells, but it was not clear whether any of them represented the type III TGF-β_1 receptor. In NRK 49F cells, the majority of bound TGF-β_1 was cross-linked to a high molecular weight complex which probably represented the type III TGF-β_1 receptor. NRK 49F cells also had type I TGF-β_1 receptors and platelet factor 4 inhibited binding to these receptors in the NRK cells. Since the type I receptor contributed only a small percentage of total TGF-β_1 binding, however, the overall effects of platelet factor 4 on TGF-β_1 binding to NRK 49F cells were negligible. We were unable to demonstrate specific or saturable binding of platelet factor 4 to Hep 3B cells using either direct binding or affinity cross-linking assays. Thus, it is not clear whether platelet factor 4 inhibits TGF-β_1 binding by competition for binding to the type I receptor.

Modest concentrations of TGF-β_1 reduced the adherence of Hep 3B cells to tissue culture dishes. Platelet factor 4 did not mimic this effect of TGF-β_1, nor did it inhibit the effect, even at concentrations which were sufficient to completely inhibit binding to the type I TGF-β_1 binding protein/receptor. This suggests that the type I binding protein does not mediate the effect of TGF-β_1 on Hep 3B cell adhesion.

Key words: growth factor receptors, heparin binding proteins, cell adherence, hepatoma cells, affinity cross-linking

Transforming Growth Factor β_1 (TGF-β_1) is believed to participate in the control of diverse biological processes (see review by Barnard et al. [1]). In vitro, TGF-β_1 has been shown to inhibit the growth of epithelial cells [2], stimulate anchorage-independent growth and extracellular matrix formation in fibroblasts [3–5], inhibit the differentiation of pre-adipocytes, pre-myelocytes, and hematopoietic stem cells [6–8], stimulate the differentiation of kidney and bronchial cells [9], and cause hypertrophy of renal proximal tubular cells [10]. In vivo, TGF-β_1 has been shown to stimulate would healing [11], inhibit liver regeneration [12], and stimulate angiogenesis [13]. There is also evidence that TGF-β_1 and its close family members, TGF-β_2 and TGF-β_3, regulate many steps in embryogenesis and tissue remodeling [1]. Given the array of potential actions for TGF-β_1, one would expect that multiple regulatory mechanisms exist to delimit both the sites and the modes of action of this growth factor. The widespread distribution of mRNA coding for TGF-β_1 in normal tissues suggests that it may act over relatively short distances [14]. TGF-β_1 is stored and secreted in a latent complex [15,16] which includes the amino terminal portion of the precursor and a separate binding protein [17]. Active TGF-β_1 can be released from this complex by treatment with acid or proteases [15,16]. TGF-β_1 may be bound and

Received January 7, 1991; accepted April 15, 1991.

inactivated in serum by α-2-macroglobulin as part of a disposal mechanism [18]. These factors may all serve to direct the actions of TGF-β_1 toward discreet targets.

The responses of target tissues to TGF-β_1 may be also be determined by the presence or absence of specific cell-surface receptors for TGF-β_1. Most normal cell types have cell surface binding proteins which recognize TGF-β_1 with high affinity and specificity [15,19–22; reviewed by Massagué et al. (33)]. In affinity cross-linking studies, it has been shown that it is common for a single cell type to express multiple TGF-β_1 binding proteins [20–22]. These binding proteins are distinguishable by their molecular weights, their relative affinities for TGF-β_1 and TGF-β_2, and their polysaccharide components [20–23]. To date, it has not been unequivocally demonstrated that any of these binding proteins act as true receptors, in the sense that they generate the intracellular signals which mediate TGF-β_1 action. There is, however, correlative evidence which links the occupancy of specific binding proteins to specific actions of TGF-β_1 [8,22,24]. Thus, it is possible that different TGF-β_1 receptors mediate different actions of TGF-β_1. If this is true, then the presence of a specific receptor type on the surface of a cell could direct it to respond to TGF-β_1 in a precise manner. The availability of agents which specifically block TGF-β_1 binding to specific receptors could be useful in addressing these issues.

Platelets are among the richest sources of TGF-β_1 [22,25]. In addition to TGF-β_1, platelets contain large amounts of platelet factor 4 and other "anti-heparin" proteins, as well as PDGF, other growth factors, and a variety of cell adhesion proteins [26–28]. These proteins are all found in the platelet α granule and are released together during platelet degranulation [26–29]. Presumably, the release of growth factors at a site of injury aids in the wound-healing process. Their close physical proximity suggests that the platelet proteins may interact functionally as well. In this paper, we report the surprising finding that platelet factor 4 selectively inhibits the binding of TGF-β_1 to a low molecular weight TGF-β_1 binding protein.

METHODS

The human hepatocellular carcinoma line Hep 3B and the normal rat kidney fibroblast line NRK 49F were obtained from the American Type Culture Collection. NRK 49F cells were grown in Dulbecco's MEM, containing 10% normal calf serum. Hep 3B cells were grown in MEM supplemented with 10% fetal calf serum. Both cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2.

TGF-β_1 was purified according to the method of Assoian et al. [25] from outdated human platelets donated by the American Red Cross. Acid/ethanol extracts of the washed platelets were applied to a 16 × 100 cm Biogel P60 column which was equilibrated and eluted with 1M acetic acid. TGF-β_1 was detected in the column fractions by testing aliquots for their ability to inhibit the binding of ^{125}I-labeled TGF-β_1 to intact cells, as described below. Aliquots of the fractions were also run on 12.5% polyacrylamide gels which were then stained with silver to reveal the 25,000 dalton TGF-β_1 homodimer. Peak fractions were pooled, lyophilized, and resuspended in 1M acetic acid/8M urea. The pooled material was then applied to a 2.5 × 100 cm Biogel P60 column which was eluted with 1M acetic acid/8M urea. Small aliquots (0.5–1.0 μl) of the fractions were assayed for TGF-β_1 using the competitive radioligand binding assay. In order to neutralize the acetic acid, the column samples were treated as follows: 2.5–5.0 μl aliquots of the column fractions were added to 125 μL of 2× binding buffer containing 2% bovine serum albumin (BSA) 2.5–5.0 μL of 1N NaOH was then added and the final volume was adjusted to 250 μL with water. 50 μL of the resulting mixture was then added to the assay. The additional salt did not appreciably affect the results of the assay. Nonetheless, in the data presented in Figure 1 control binding was determined in quadruplicate assays in which aliquots of the 8M urea, 1 M acetic acid elution buffer was treated in the same manner and added to the binding reaction.

Additional purification on a C_{18} reversed phase HPLC column [30] was carried out after the final step of the Assoian procedure. The final product was greater than 95% pure as assessed by silver stained polyacrylamide gels. The concentration of the purified TGF-β_1 was calculated from amino acid analysis.

TGF-β_1 was labeled with ^{125}I according to the chloramine T method of Frolik et al. [19]. The labeled growth factor was purified either by gel filtration on superfine Sephadex G-25 columns or by HPLC on reversed phase C_{18} columns. There was no appreciable difference in the binding activity of TGF-β_1 purified using these two

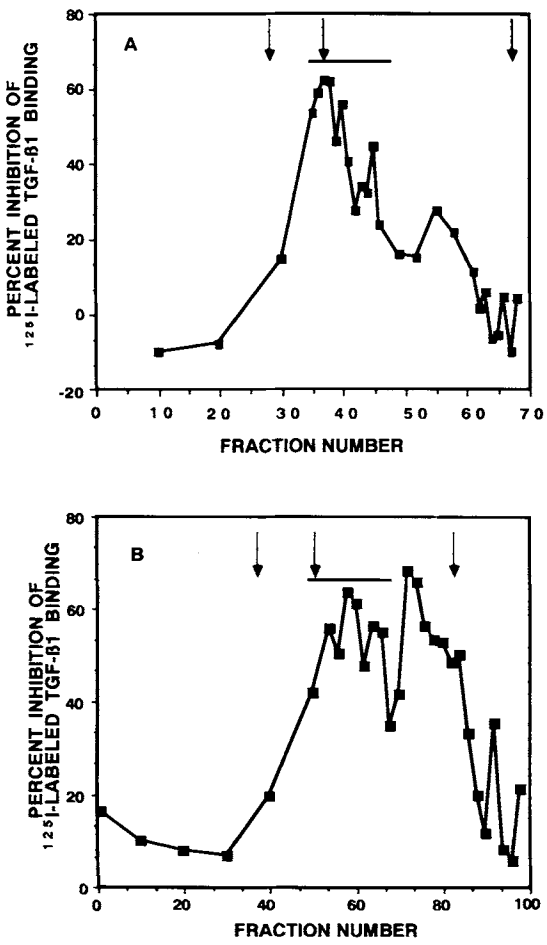

Fig. 1. Inhibition of binding of ^{125}I-labeled TGF-β_1 to NRK 49F and Hep 3B cells by Biogel P60 fractions of human platelet extracts. A: Human platelet extracts were eluted through a single Biogel P60 column in the presence of 1M acetic acid and the TGF-β_1-containing peak fractions were lyophilized and applied to second Biogel P60 column and eluted with 1M acetic acid and 8M urea. Aliquots of the indicated fractions were treated as described in Methods and tested for their ability to inhibit binding of ^{125}I-labeled TGF-β_1 to NRK 49F cells. B: A separate platelet extract was partially purified as in A and applied to a second urea-containing Biogel P60 column. Aliquots of the indicated fractions were tested for their ability to inhibit binding of TGF-β_1 to Hep 3B cells. In both A and B, vertical arrows indicate from left to right the positions of molecular weight standards bovine serum albumin (68,000 daltons) chymotrypsinogen A (24,500 daltons) and lysozyme (14,400 daltons). The horizontal bars indicate fractions which had the most intense silver-staining for the 25,000 dalton TGF-β_1 band on 15% polyacrylamide gels.

methods. The specific activity of the labeled products varied from 60–150 μCi/μg.

Platelet factor 4 was purified on C_{18} HPLC columns as follows: The peak fractions from a biogel P60 column (see Results) were pooled, dialyzed against 1M acetic acid, and lyophilized and redissolved in 4 mM HCl. This was injected onto a 1 × 20 cm C_{18} column which was equilibrated with 0.1% trifluoroacetic acid in water. The column was washed with this solvent for 15 min at a flow rate of 1.5 ml/min. The column was then washed with 14% acetonitrile for 20 min and platelet factor 4 was eluted from the column with gradient of 14–70% acetonitrile over 45 min. Platelet factor 4 was iodinated using chloramine T and the labeled protein was purified on a C_{18} column. For some experiments (Fig. 8C) commercial platelet factor 4 (Sigma) was dissolved in 1M acetic acid and further purified by HPLC in the same manner.

Competitive binding assays were performed as follows: The cells to be used for binding were grown to near confluency under standard conditions in 24-well plates. The medium was replaced with standard medium containing 1% serum 18 hours before the binding assays. The cells were washed 4 times with 1 ml of ice-cold binding buffer (50 mM HEPES, pH 7.5, plus 5 mM $MgSO_4$, 128 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, and 0.1% BSA). Binding incubations were carried out at 15°C in a total volume of 0.3 ml of binding buffer containing 50,000–100,000 CPM of ^{125}I-labeled TGF-β_1 and varying amounts of either unlabeled TGF-β_1 or other competitors. At the end of the incubation, the cell monolayers were washed 4 times with 1 ml of ice-cold Hanks balanced salt buffer containing 0.1% BSA. The cells were then solubilized for 20 min at room temperature in 10 mM Tris, pH 7.0, containing 10% glycerol, 0.01% BSA, and 1.0% Triton X100. The solubilized extract was then counted in a gamma counter.

Affinity cross-linking studies were done according to the method of Massagué and Like [20]. Cell lines were grown to 70–90% of confluency in their normal medium, then incubated in medium containing 1% serum 18 hours before assay. The monolayer cultures were washed 4 times in binding buffer. Cells were then incubated in binding buffer with 0.1% BSA containing 20–50 pM ^{125}I-labeled TGF-β_1 in the presence of 0–10,000 pM native TGF-β_1. After a 4 hour incubation at 4°C, the cells were washed 4 times in Hanks balanced salt buffer containing 0.1% BSA. Bound TGF-β_1 was then cross-linked to its receptors by incubating the cells with 0.5 mM disuccinimidyl suberate in Hanks buffer for 20 min on ice. The cells were then washed once with Hanks buffer. The cells were scraped from the wells in 10 mM Tris, pH 7.0, containing 1

mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 10 μg/ml of leupeptin, and 0.25 M sucrose. The cells were transferred to micro centrifuge tubes and pelleted at 10,000*g* for 10 minutes. The cell pellets were resuspended in 10 mM Tris buffer at pH 7.0 containing 1 mM EDTA, 1.0% Triton X100, 0.1 mM PMSF, 10 μg/ml pepstatin, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 50 μg/ml aprotinin, 10 μg/ml antipain, and 0.5 mM benzamidine. This suspension was mixed for 45 min at 4°C and re-centrifuged. The solubilized receptors were then removed and added to an equal volume of electrophoresis sample buffer (10 mM phosphate buffer with 4% SDS, 8 M urea, 0.2% bromophenol blue, and 0.2 M dithiothreitol), boiled, and counted in a gamma counter. The samples were then frozen at −70°C until they were applied to polyacrylamide gels. Samples were applied to 9% polyacrylamide gels or 5–10% gradient gels made according to the method of O'Farrell [31]. The gels were stained with Coomassie Blue, de-stained, and dried. The gels were then exposed to Kodak XAR5 x-ray film for 1–10 days to reveal the TGF-β_1 binding complexes.

The effects of platelet factor 4 and TGF-β_1 on the growth of Hep 3B cells were assessed as follows: Confluent cultures were trypsinized and passed at 1:10 in their normal medium. Initial cell density was 0.39×10^4 cells/cm^2. After allowing the cells to adhere for three hours, the growth factors were applied at the indicated concentrations. After four days, the cells were again trypsinized and counted.

RESULTS

In the TGF-β_1 purification procedure of Assoian et al. [25] platelet extracts are first applied to a Biogel P60 column in 1M acetic acid. TGF-β_1 runs anomalously on this column, eluting with lower molecular weight proteins. The TGF-β_1-containing fractions from this column are then applied to a Biogel P60 column which is equilibrated and run in 1M acetic acid containing 8M urea. Under these conditions TGF-β_1 runs according to its true molecular weight and is separated from the contaminating low molecular weight proteins [25]. In our purifications of TGF-β_1, we have used a competitive radioligand binding assay to detect TGF-β_1 in the fractions of the urea-containing column. When aliquots of the column fractions were tested for their ability to compete with ^{125}I-labeled TGF-β_1 for binding to NRK 49F fibroblasts, there was a single peak of competing activity (Fig. 1A). This peak eluted near the position expected for TGF-β_1 on the basis of molecular weight markers. When the proteins in the peak fractions were resolved on 12.5% polyacrylamide gels it was seen that the most abundant component had a molecular weight of 25 kDa as expected for the TGF-β_1 homodimer. Final purification on HPLC and amino acid analysis confirmed that the 25 kDa protein was in fact TGF-β_1 (data not shown).

In contrast, when cells of the hepatocellular carcinoma line Hep 3B were used in the binding assay, a second peak of competing activity was detected. Fig. 1B shows the profile obtained when a separate partially purified platelet extract (through the first biogel P-60 column) was applied to a urea-containing biogel P60 column and aliquots of the fractions were allowed to compete with ^{125}I-labeled TGF-β_1 for binding to Hep 3B cells. In addition to the TGF-β_1 peak, a lower molecular weight peak was detected. Analysis of the second peak fractions on polyacrylamide gels indicated that the major protein component in these fractions had a molecular weight of less than 14 kDa. When subsequent platelet extracts were analyzed in this way, the relative heights of the TGF-β_1 peak and the lower molecular weight peak varied considerably, so that differences between the column profiles shown in Figs. 1A and B may be due, in part, to variation among the extracts. Nonetheless, further analyses revealed that differences in the complements of TGF-β_1 receptors found in Hep 3B cells and NRK 49F cells make the lower molecular weight activity more readily detectable by Hep 3B cells (see below).

When the low molecular weight material was applied to a C_{18} HPLC column, several proteins were resolved in the acetonitrile gradient, but only one peak had a measurable ability to inhibit the binding of ^{125}I-labeled TGF-β_1 to Hep 3B cells (Fig. 2A). Since this peak was not completely resolved, the peak fractions were pooled and concentrated and half of the material was re-applied to the same column. This gave a well-resolved absorbance peak which coincided with the TGF-β_1-binding inhibitory activity (Fig. 2). Analysis of the peak fractions on a silver-stained polyacrylamide gel indicated the presence of a low molecular weight protein which was greater than 95% pure. A faint band with an apparent molecular weight of ~20 kDa is visible in fraction 30.

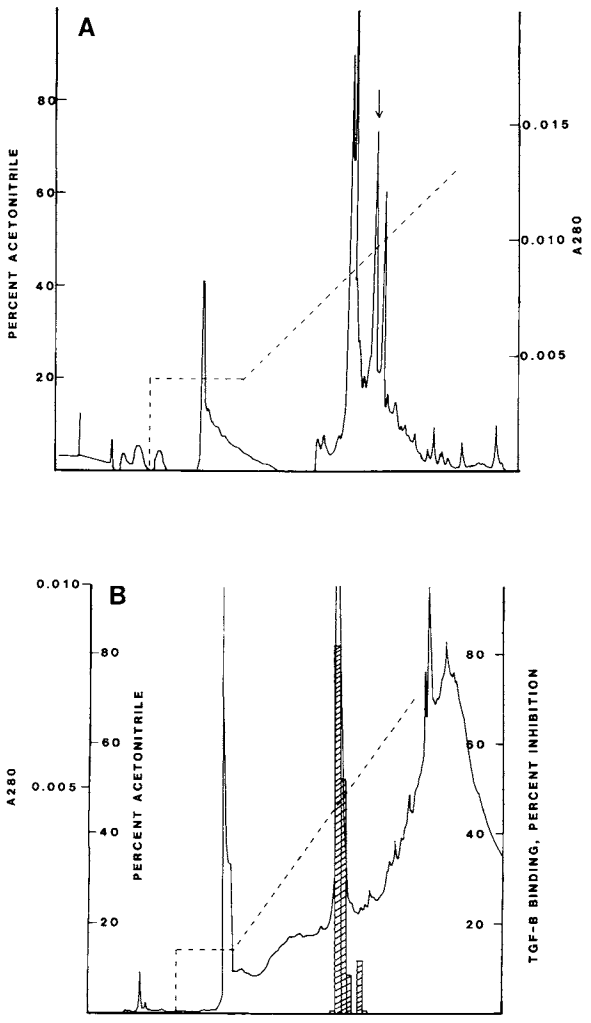

Fig. 2. HPLC purification of the low molecular weight TGF-β_1 binding inhibitor. **A:** Fractions 71–76 from the column shown in Fig. 1B were pooled, dialyzed against 4mM HCl, and applied to a C_{18} reversed phase HPLC column, which was developed with an acetonitrile gradient. Column fractions were tested for their ability to inhibit binding of ^{125}I-labeled TGF-β_1 to Hep 3B cells. The peak inhibiting fraction is indicated with an arrow. **B:** The peak fraction was vacuum concentrated, re-applied to the C_{18} column, and eluted in the same manner. The cross-hatched bars indicate inhibition of TGF-β_1 binding to Hep 3B cells.

TABLE I. Amino Acid Sequencing of Low Molecular Weight TGF-β_1 Binding Inhibitor*

Cycle	Amino acid detected	Platelet factor 4 sequence[a]
1	GLU	GLU
2	ALA	ALA
3	GLU	GLU
4	GLU	GLU
5	ASP	ASP
6	GLY	GLY
7	ASP	ASP
8	LEU	LEU
9	GLN	GLN
10	CYS	CYS
11	LEU	LEU
12	ND[b]	CYS
13	VAL	LYS
15	THR	THR
16	THR	THR
17	SER	SER
18	GLN	GLN
19	VAL	VAL
20	ARG	ARG
21	PRO	PRO
22	ND[b]	ARG
23	HIS	HIS
24	ILE	ILE
25	THR	THR
26	SER	SER
27	LEU	LEU
28	GLU	GLU
29	VAL	VAL
30	ILE	ILE
31	LYS	LYS
32	ALA	ALA
33	GLY	GLY
34	PRO	PRO
35	HIS	HIS

*The peak fraction in Fig. 2 was sequenced using an automated Edman procedure.
[a]As reported in ref. 35.
[b]ND, not determined.

To determine its identity, the inhibitory protein was sequenced via an automated Edman procedure. Thirty-two of the first thirty-five amino-terminal residues gave an unambiguous match for the sequence of human platelet factor 4 (Table I).

To examine the differences between the TGF-β_1 binding proteins of Hep 3B and NRK 49F cells, we used purified platelet factor 4 and TGF-β_1 in competition assays with ^{125}I-labeled TGF-β_1. In Hep 3B cells, unlabeled TGF-β_1 inhibited the binding of ^{125}I-labeled TGF-β_1 with an ED50 of approximately 5×10^{-10}M (Fig. 4). By contrast, the TGF-β_1 competition curve for NRK 49F cells was much shallower ($ED_{50} = 5 \times 10^{-9}$ M) (Fig. 4). Purified platelet factor 4 inhibited the binding of TGF-β_1 to Hep 3B cells quite effectively (Fig. 5A) but relatively high concentrations were required ($ED_{50} = 5 \times 10^{-8}$ M). Platelet factor 4 was a very poor inhibitor of TGF-β_1 binding to NRK 49F cells (Fig. 5B).

In both cell types, the effects of platelet factor 4 on TGF-β_1 binding were similar regardless of

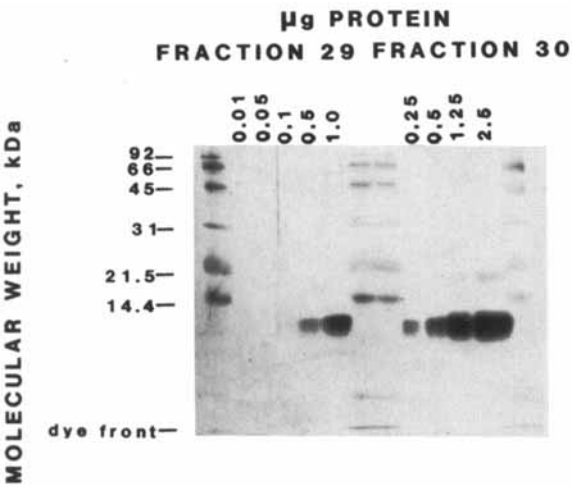

Fig. 3. Polyacrylamide gels of purified low molecular weight TGF-β_1 binding inhibitor. Increasing concentrations of protein from the peak fractions of the HPLC column depicted in Fig. 2B were resolved on a 15% polyacrylamide gel which was then silver stained. The µg of protein applied to each lane are indicated. The protein concentrations in the fractions were 40.8 µg/ml for fraction 29 and 23.5 µg/ml for fraction 30. Lanes 1, 7, 8, and 13 are molecular weight standards.

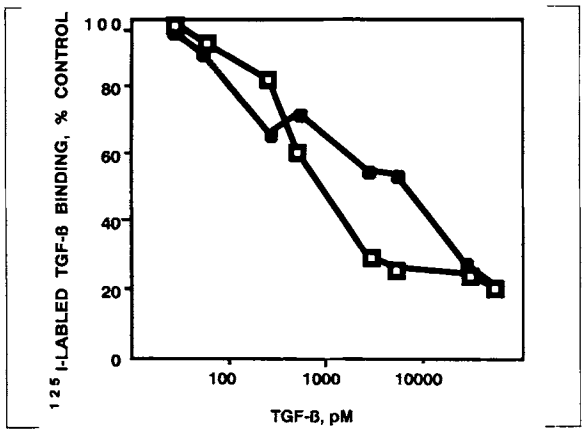

Fig. 4. TGF-β_1 binding competition curves for Hep 3B and NRK 49F cells. ^{125}I-labeled TGF-β_1 was incubated with Hep 3B cells (open squares) or NRK 49F cells (closed circles) in the presence of 27, 54, 270, 540, 2,700, 5,400, 27,000 and 54,000 pM unlabeled TGF-β_1. Points represent means of duplicate determinations which varied by less than 5%.

whether it was purified as described above or obtained from a commercial source which used a completely different method of purification (Fig. 5A,B). Our HPLC-purified platelet factor 4 appeared to be slightly more efficient than the commercial product in inhibiting TGF-β_1 binding to Hep 3B cells (Fig. 5A). Since the commercial preparation used here was not homogeneously pure, however, we may have overestimated its concentration.

Many cell types have three distinct TGF-β_1 binding proteins and the cell-surface concentration of a particular binding protein varies considerably from one cell type to the next [33]. As a result, a given protein may account for significant TGF-β_1 binding in one cell type and little or no binding in another [20,21,24]. We reasoned that such variation could account for the differences between the competition curves for Hep 3B and NRK 49F cells. In order to test this idea and to identify which TGF-β_1 binding proteins were inhibited by platelet factor 4, we performed affinity cross-linking assays. Figure 6 (right) shows the results of cross-linking ^{125}I-labeled TGF-β_1 to NRK 49F cells after incubating them in the presence of various concentrations of competing unlabeled TGF-β_1. It can be seen that in these cells, the majority of TGF-β_1 binds to a high molecular weight protein which barely enters the resolving gel. This protein has been identified as a proteoglycan by others [23,32]. There are also two fainter bands on the autoradiograph which migrate at 90 and 70 kDa. These bands presumably correspond to binding proteins of 78 and 58 kDa, allowing for cross-linking of a single chain of the TGF-β_1 dimer. These 90 and 70 kDa bands are abolished at lower concentrations of competing TGF-β_1 than the high molecular weight band, suggesting that the binding proteins they represent have a higher affinity for TGF-β_1. The greater intensity of the high molecular weight band suggests that the high molecular weight binding protein is more abundant than the smaller proteins on NRK 49F cells. The fact that this low affinity binding protein binds the majority of TGF-β_1 accounts for the overall low affinity of TGF-β_1 binding to the intact cells. Similar results have been seen by others in cross-linking studies with NRK 49F cells [20,21].

The pattern of TGF-β_1 binding proteins in Hep 3B cells is strikingly different from that of NRK 49F cells (Fig. 6, left). In these cells, the most intense binding signal comes from a broad band of roughly 70 kDa which appears to correspond to the 70 kDa band of NRK 49F cells. There is also a faint band of approximately 80 kDa which represents a high-affinity TGF-β_1 binding protein of 68 kDa. This 68 kDa protein may correspond to the 78 kDa protein of NRK 49F cells (see Discussion). In addition there are numerous minor bands of higher molecular

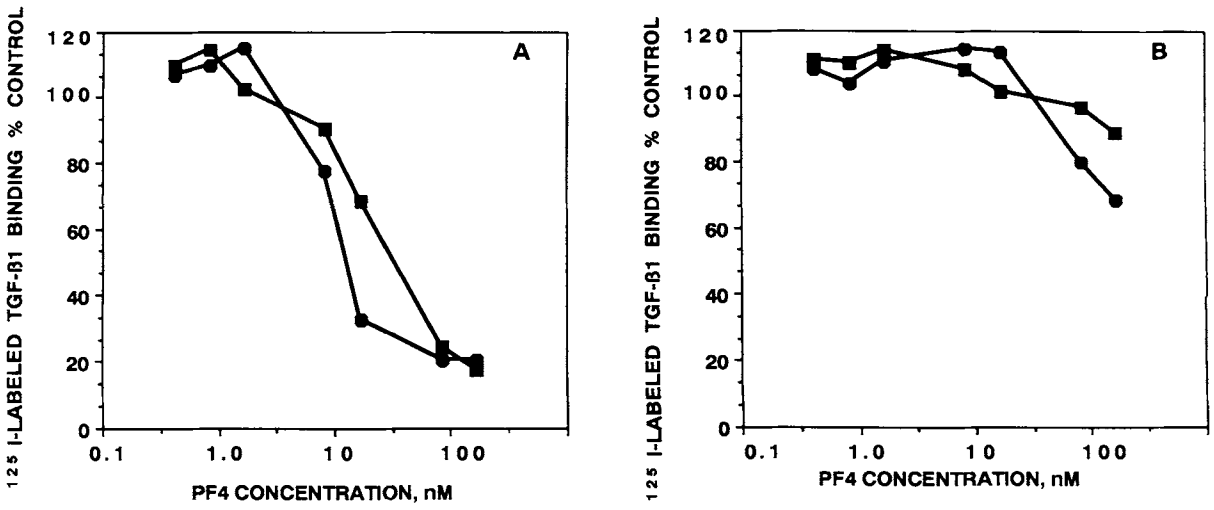

Fig. 5. Inhibition of binding of ^{125}I-labeled TGF-β_1 to Hep 3B cells and NRK 49F cells by platelet factor 4. ^{125}I-labeled TGF-β_1 was incubated with either Hep 3B cells (**A**) or NRK 49F cells (**B**) in the presence of the indicated concentrations of platelet factor 4. Squares represent commercially prepared platelet factor 4 and circles represent platelet factor 4 purified by HPLC as described above.

weight. It is not clear that any of these corresponds to the high molecular weight binding protein of NRK 49F cells. Thus, both the kinds and the concentrations of TGF-β_1 binding proteins of Hep 3B cells differ from those of NRK 49F cells. The fact that the dominant binding protein in Hep 3B cells has a higher affinity for TGF-β_1 than the dominant protein of NRK 49F cells could account for the higher overall affinity of TGF-β_1 binding in the Hep 3B cells.

We also performed affinity cross-linking experiments in the presence of platelet factor 4 to determine which of the TGF-β_1 binding proteins was affected by this inhibitor. Platelet factor 4 inhibited the binding of mainly the protein in the 70 kDa band in the NRK 49F cells (Fig. 6). Labeling of the high molecular weight band was slightly stimulated by low concentrations of platelet factor 4 and only slightly inhibited at the highest concentrations. The relatively small contribution of the 70 kDa band protein to TGF-β_1 binding in these cells would account for the poor overall inhibition of TGF-β_1 binding by platelet factor 4 in intact NRK 49F cells.

For the Hep 3B cells, we used gradient gels of 5–10% acrylamide to improve the resolution of the TGF-β_1 binding proteins (Fig. 7). Under these conditions we could resolve multiple ^{125}I-labeled complexes. The 70 kDa band seen in Figure 6 is now resolved into 3 bands of approximately 75, 70, and 67 kDa. The high affinity binding complex seen in Figure 6 (80 kDa band) migrates as a diffuse band of somewhat higher molecular weight in this gel system (~ 90 kDa). Platelet factor 4 most strongly inhibits binding to the protein in the 70 kDa band. It also inhibits its binding to the proteins in bands of 75 kDa, 130 kDa, and the diffuse band at the top of the gel. Platelet factor 4 is relatively inefficient in inhibiting binding to proteins at 67, 90, 180, and 220 kDa. Interestingly the protein with the highest affinity for TGF-β_1 (in the 90 kDa band) was least sensitive to inhibition by platelet factor 4. Conversely the TGF-β_1 binding protein most strongly inhibited by platelet factor 4 had a relatively low affinity for TGF-β_1. The fact that platelet factor 4 inhibits binding to the most intensely stained TGF-β_1 binding protein in the Hep 3B cells probably accounts for the high degree to which platelet factor 4 inhibits binding to these cells.

It is not clear from the preceding experiments whether platelet factor 4 actually competes for binding to the 70 kDa binding complex or inhibits its TGF-β_1 binding by another means. We therefore attempted to demonstrate specific binding of ^{125}I-labeled platelet factor 4 to these cells. Although up to 12% of added ^{125}I-labeled platelet factor 4 bound to Hep 3B cells, the binding was non-saturable; no ^{125}I was displaced by a 1,000-fold excess of unlabeled platelet factor 4. We next attempted to demonstrate specific binding using affinity cross-linking. We found no evi-

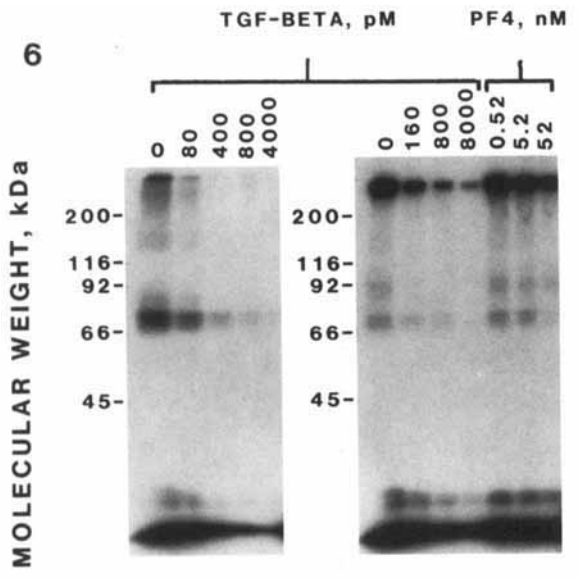

Fig. 6. Cross-linking of ^{125}I-labeled TGF-β_1 to Hep 3B cells and NRK 49F cells. Hep 3B cells (**left panel**) or NRK 49F cells (**right panel**) were incubated with ^{125}I-labeled TGF-β_1 in the presence of either unlabeled TGF-β_1 or platelet factor 4, and the bound TGF-β_1 was cross-linked to the cells as described in Methods. The cells were then lysed and the cross-linked proteins were resolved on 9% polyacrylamide gels which were then dried and autoradiographed. Results shown are representative of multiple experiments.

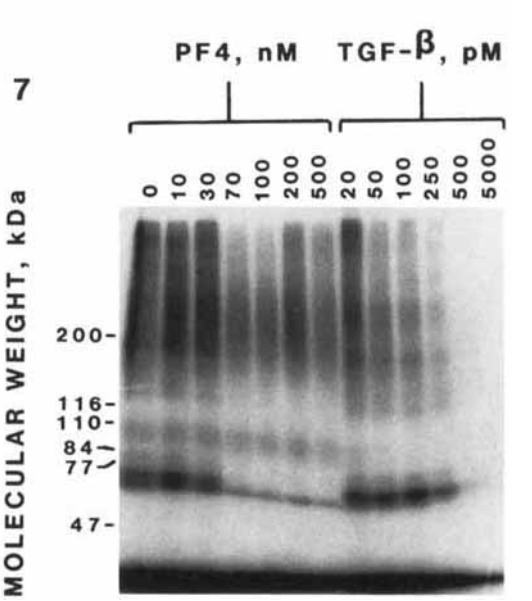

Fig. 7. Cross-linking of TGF-β_1 to Hep 3B cells. ^{125}I-labeled TGF-β_1 was incubated with Hep 3B cells in the presence of the indicated concentrations of unlabeled TGF-β_1 or platelet factor 4. Bound TGF-β_1 was cross-linked to the cells which were than lysed as described in Methods. Triplicate lysates for each condition were pooled, and aliquots of the pooled material were applied to a 5–10% linear polyacrylamide gel. The labeled proteins were then resolved and autoradiographed. Results are representative of 3 separate experiments.

dence of specific binding of platelet factor 4 to either Hep 3B or NRK 49F cells.

We next considered the effects of platelet factor 4 and TGF-β_1 on Hep 3B cell growth. TGF-β_1 affects the adhesion of these cells to tissue culture dishes. When TGF-β_1 is added to low density cultures of Hep 3B cells, the dividing cells detach from the tissue culture dishes and float. After several rounds of division, there is a substantial reduction in the number of adherent cells in TGF-β_1-treated vs. control cultures (Fig. 8A). This phenomenon occurs at very modest TGF-β_1 concentrations, with significant effects detectable at 20 pM. This does not appear to be a toxic effect, since the cells which remain adherent incorporate ^{3}H-Thymidine at the same rate as control cells (data not shown). Platelet factor 4 did not inhibit this action of TGF-β_1. In fact, high concentrations (500 nM) of a relatively crude commercial preparation of platelet factor 4 actually enhanced the effects of TGF-β_1 somewhat (Fig. 8B). No alterations of the effects of TGF-β_1 were seen in the presence of 100 nM platelet factor 4 (not shown). In order to see whether platelet factor 4 alone mimics the effects of TGF-β_1 on Hep 3B cells, we added HPLC-purified commercial platelet factor 4 to low density cultures. It can be seen that even at the highest concentrations of platelet factor 4, its effects on cell adhesion do not approach those of TGF-β_1 (Fig. 8C). At a concentration of 100 nM, which is sufficient to completely inhibit the binding of TGF-β_1 to the 70 kDa binding complex (see Fig. 7) there is no detectable effect on cell adhesion.

DISCUSSION

In purifying TGF-β_1 from human platelets, most workers have used its effects on cell growth as an assay [25,30]. Thus, it is not surprising that the effects of platelet factor 4 on TGF-β_1 binding have not been reported previously. In fact, even when column fractions were assayed with a competitive radioligand binding assay, inhibition by platelet factor 4 was readily detectable only with Hep 3B cells (Fig. 1A,B). In this study, platelet factor 4 was purified to homogeneity solely on the basis of its ability to inhibit the binding of TGF-β_1 to Hep 3B cells. Several factors give us confidence that the inhibition of TGF-β_1 binding is a property of platelet factor 4, and is not due to an undetected contamination with TGF-β_1. First, we saw no trace of contaminating TGF-β_1 in our purified platelet factor 4 after either silver staining (Fig. 3) or iodination.

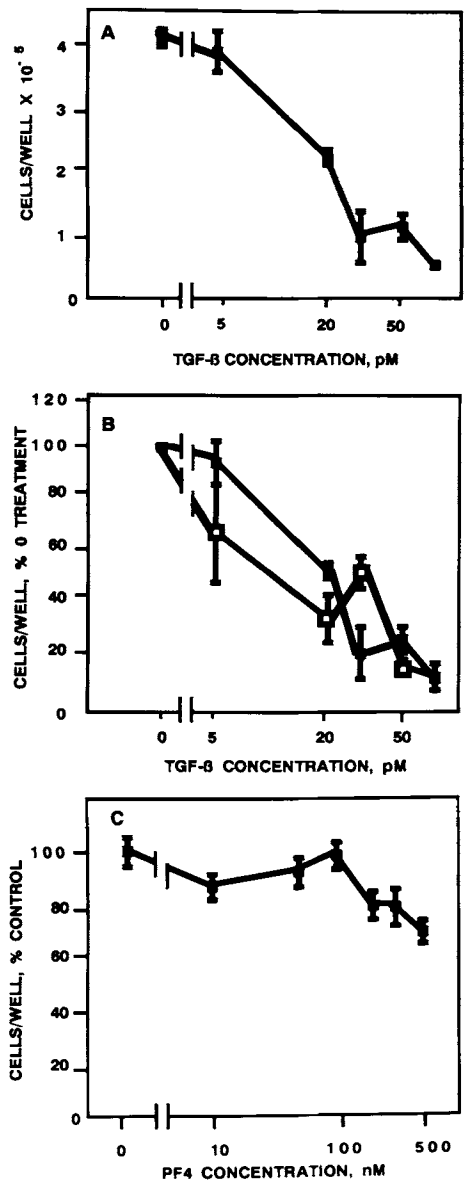

Fig. 8. Effects of TGF-β_1 and platelet factor 4 on the adherence of Hep 3B cells. Confluent cultures of Hep 3B cells were passed at 1:10 into 6-well tissue culture plates and the indicated concentrations of TGF-β_1 or platelet factor 4 were added. After 4 days in culture the adherent cells were trypsinized and counted. **A:** Dose response for TGF-β_1 alone. Data shown are means, plus or minus standard deviation of duplicate counts of triplicate wells. **B:** TGF-β_1 dose response as in A in the presence (open squares) or absence (closed squares) of 500 nM crude platelet factor 4. **C:** Cells were incubated as above with HPLC-purified platelet factor 4. Data represent means, plus or minus standard deviations of quadruplicate counts of triplicate wells. Control cell densities were 4.28 ± 0.15 × 10^4 cells/cm^2 for A and B, and 1.5 × 10^4 cells/cm^2 for C.

Second, we were able to demonstrate inhibition of TGF-β_1 binding both with our preparation and with a commercial preparation of platelet factor 4 (Fig. 5). Third, platelet factor 4 inhibited binding principally to specific TGF-β_1 binding proteins and was least effective in inhibiting binding to the protein with the highest affinity for TGF-β_1 (Fig. 7). These results would not be expected if our preparation were contaminated with TGF-β_1.

We have shown that platelet factor 4 inhibits the binding of TGF-β_1 to Hep 3B cells better than it inhibits the binding of TGF-β_1 to NRK 49F cells (Fig. 5A,B), and this difference appears to result from differences in the complements of TGF-β_1 binding proteins found on these two cell types (Figs. 6, 7). In NRK 49F cells, binding is dominated by a low affinity high molecular weight protein (Fig. 6 and ref. 20) which has been shown by others to be a proteoglycan [24,31,33]. TGF-β_1 binding to this protein is inhibited very little by platelet factor 4. In Hep 3B cells, TGF-β_1 binding is dominated by a moderate affinity protein which appears as a 70 kDa complex in affinity cross-linking studies. Binding to this protein is strongly inhibited by platelet factor 4. Based on its size and affinity for TGF-β_1, we believe that the 70 kDa band in Hep 3B cells is the protein referred to by Massagué et al. [33] as the type I TGF-β_1 receptor. This protein was also seen in NRK 49F cells in our studies and in many other cell types in the studies of Massagué and Cheifetz et al. [20–22]. Platelet factor 4 also inhibited labeling of the 70 kDa band in NRK 49F cells, strengthening our belief that the two binding proteins are the same. Hep 3B cells also appear to have the high affinity TGF-β_1 binding protein referred to by Massagué et al. as the type II TGF-β_1 receptor [33]. As we have observed and as reported by Massagué et al. [33], this binding complex migrates with slightly different molecular weights in cells from different species (92 kDa in rat NRK 49F cells, 80 kDa in human Hep 3B cells).

It is not clear whether Hep 3B cells have an equivalent of the proteoglycan TGF-β_1 binding protein which is found on NRK 49F cells and referred to as the type III TGF-β_1 receptor by Massagué et al. [33]. Hep 3B cells have several high molecular weight TGF-β_1 binding proteins, but none of them exactly match the molecular weight or the binding affinity of the NRK 49F high molecular weight binding protein. It is not possible to correctly estimate the molecular weight of the NRK 49F protein from the gel shown in Figure 6, since the protein does not enter the resolving gel. When the cross-linked binding protein is run on a 5–10% gradient gel, however, it runs as a diffuse band with an appar-

ent average molecular weight of 280 kDa [Whitson, unpublished results, 23,32].

The large number of bands seen in the Hep 3B cross-linking experiments was surprising, although it was quite reproducible. It seems very unlikely that all the bands represent bona fide TGF-β_1 receptors or even TGF-β_1 binding proteins. It is possible that some of the bands represent fragments of larger proteins which are generated by proteolysis during the lysis of Hep 3B cells, but not NRK 49F cells. The pattern of labeled complexes at 65–75 kDa in Hep 3B cells resembles that seen in 3T3-L1 cells which have been treated with trypsin after affinity labeling [23]. Trypsin treatment of 3T3L1 cells removes the proteoglycan portion of the type III receptor and generates bands of 72, 63, and 60 kDa. Despite the presence of numerous protease inhibitors in our solubilization buffer, the liver-derived Hep 3B cells may release active proteases which attack the type III binding protein. This would explain both the numerous labeled bands on the cross-linking gels and the apparent absence of the type III binding protein (Fig. 7). NRK 49F fibroblasts may lack the inhibitor-resistant proteases. Cross-linked TGF-β_1 binding proteins of NRK 49F cells show only one band at 70 kDa when run on 5–10% gradient gels (not shown).

Moderate concentrations of unlabeled competing TGF-β_1 eliminate the ^{125}I-labeling of the Hep 3B bands indicating that binding to the proteins they represent is at least saturable. Also, the overall affinity of TGF-β_1 binding which can be deduced from the cross-linking experiments with both Hep 3B and NRK 49F cells is consistent with that observed in conventional binding assays to the intact cells.

We were unable to demonstrate saturable binding of platelet factor 4 to Hep 3B cells either in direct assays or cross-linking experiments. Since platelet factor 4 binds strongly to heparin, it is possible that interactions with heparan sulfate in cell-surface proteoglycans mask binding to a specific receptor. This is known to occur in binding assays for the heparin binding proteins acidic and basic FGF and can be eliminated by including free heparin in the binding assays [34]. Our attempts to demonstrate specific binding of platelet factor 4 to Hep 3B cells in the presence of free heparin also failed, however (data not shown). Thus, it is not clear whether platelet factor 4 actually competes with TGF-β_1 for binding to the 70 kDa binding complex. There is virtually no sequence homology between TGF-β_1 and platelet factor 4 [14,35], although there is some similarity in their hydropathy plots.

One possible alternative mechanism for inhibition of TGF-β_1 binding by platelet factor 4 is direct interaction between the two proteins. In order to account for selective inhibition of binding to the 70 kDa binding complex, it is then necessary to propose that platelet factor 4 masks only the part of the TGF-β_1 dimer which interacts with this binding protein, leaving the rest of the molecule free to interact with other binding proteins. This would imply that the various TGF-β_1 binding proteins recognize different parts of the TGF-β_1 ligand. As far as we are aware, this possibility has not been addressed experimentally.

We have shown that low concentrations of TGF-β_1 cause Hep 3B cells to lose adherence. This does not seem to be a toxic effect, but rather is associated with cell division. The loss of adherence does not begin until 24 hours after the addition of TGF-β_1 and increases with time of exposure. We cannot rule out the possibility that TGF-β_1 causes the accumulation of a substance which reaches toxic levels in the cells over time. Given the potent effects of TGF-β_1 on extracellular matrix and adhesion proteins [1,3–5], it seems more likely that the loss of adherence stems from changes in these proteins. The expression of specific TGF-β_1 binding proteins and the nature of the responses to TGF-β_1 have been reported to change with cell density in some cell lines [37,38, see below]. The effects of TGF-β_1 on the adherence of Hep 3B cells appears to be independent of cell density, however, inasmuch as it occurs whether TGF-β_1 is added to cells at either 10% or 40% of confluent density (Whitson, unpublished data).

Platelet factor 4 neither mimics nor inhibits the effects of TGF-β_1 on cell adhesion, even at concentrations which are more than sufficient to inhibit binding of TGF-β_1 to the 70 kDa binding complex. Thus, it is unlikely that the binding protein in the 70 kDa complex is the receptor which mediates this effect. Given the low doses at which TGF-β_1 affects Hep 3B adhesion, it seems most likely that the high affinity 80–90 kDa TGF-β_1 binding protein is the biologically relevant receptor. However, none of the other Hep 3B TGF-β_1 binding proteins can be eliminated on the basis of the current evidence.

The biological significance of our observations is not clear. Both TGF-β_1 and factor 4 are stored in platelet alpha granules and both may be re-

leased at the site of injury during wound healing [27,29,30]. Factor 4 is released from the platelets as part of a large complex which consists of a proteoglycan which is noncovalently bound to a tetramer of the 7,700 dalton factor 4 chains. The large proteoglycan dissociates from the tetramer at high ionic strength, and the tetramer itself dissociates at acidic pH [reviewed in 34,35]. Interestingly, TGF-β_1 is released from platelets and other cells as a latent complex and this complex is also dissociated by acidic pH [15,16]. It is not clear how the two latent complexes may interact, or whether the active TGF-β_1 dimer encounters unmasked platelet factor 4 in vivo. The type I TGF-β_1 binding protein is present on a variety of normal cell types [20–22,32] but the impact of the inhibition of TGF-β_1 binding to this protein depends to some extent on whether this protein is, in fact, a TGF-β_1 receptor.

There is strong evidence that the type I binding protein is the receptor which mediates the growth-inhibiting effects of TGF-β_1 in epithelial cells. Chemically induced mutations which led to the loss of the type I binding protein abolished the response of mink lung epithelial cells to TGF-β_1 [39]. More indirect evidence links the type I binding protein to hematopoietic stem cell differentiation [8]. It has also been noted that no cell line has been found which responds to TGF-β_1 and lacks the type I binding protein [33]. The type I binding protein does not seem to mediate the effect of TGF-β_1 on Hep 3B cells, however. This effect is dissimilar to the growth arrest caused by TGF-β_1 in epithelial cells and may therefore be mediated by a different TGF-β_1 binding protein. In vascular smooth muscle cells, growth inhibition appears to be mediated by a 75 kDa receptor which disappears at high cell densities [38]. At high densities TGF-β_1 becomes stimulatory to the cells. Thus, different TGF-β_1 binding proteins appear to mediate different responses to TGF-β_1 in this cell type.

If platelet factor 4 inhibits the binding of TGF-β_1 to a functional receptor, this would provide a further level of control on TGF-β_1 action. In this context, it is worth noting that platelet factor 4 has recently been reported to inhibit the binding of basic FGF to NIH 3T3 cells [40]. Platelet factor 4 also binds saturably to bovine aortic endothelial (BAE) cells [41] and inhibits their spontaneous migration [40]. Migration of BAE cells is believed to be regulated by basic FGF via an autocrine mechanism. Platelet factor 4 also inhibits angiogenesis [42], a process which is stimulated by both TGF-β_1 [13] and basic FGF [43]. Thus, platelet factor 4 may be a modulator of the effects of these growth factors.

ACKNOWLEDGMENTS

Platelet Factor 4 was sequenced by Ray Paxton at the City of Hope Core Sequencing Facility. The authors gratefully acknowledge the assistance of Faith Sorensen in the preparation of this manuscript. This work was supported by IDEN.

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