Transforming Growth Factor-\(\beta\) Stimulates the Expression of Fibronectin and Collagen and Their Incorporation into the Extracellular Matrix*

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We have examined whether the extracellular matrix is a biochemical target for transforming growth factor-\(\beta\) (TGF\(\beta\)). We find that TGF\(\beta\) increases the expression of the major extracellular matrix proteins, fibronectin and collagen. This effect is a general response to TGF\(\beta\) seen in primary cultures and established lines of cells from various types, normal and transformed. The relative incorporation of fibronectin and collagen into the matrix also increases in response to TGF\(\beta\). The effect of TGF\(\beta\) on fibronectin levels as characterized in chick embryo fibroblasts is rapid, selective, persistent, and specific, and involves transcriptional events; it is not mimicked by other growth factors tested. The induction of anchorage-independent growth of normal fibroblasts by TGF\(\beta\) is mimicked by fibronectin and is specifically blocked by inhibitors of fibronectin binding to its cell surface receptor. The results demonstrate a functional involvement of fibronectin in mediating cellular responses to TGF\(\beta\), and suggest a model for TGF\(\beta\) action based on the control of the extracellular matrix in target cells.

Transforming growth factor-\(\beta\) is a hormonally active polypeptide that controls cell growth and differentiation (for reviews see Sporn and Roberts, 1985; Massagué, 1985a). It is present in normal tissues as well as in transformed cells (Roberts et al., 1981). Blood platelets, in particular, store relatively large amounts of TGF\(\beta\) (Childs et al., 1982; Asooian et al., 1983) suggesting that this factor may play an important role in the normal response to tissue injury. TGF\(\beta\) from human platelets and other normal tissues (Asooian et al., 1983; Frolik et al., 1983; Roberts et al., 1983) as well as from retrovirally transformed rat cells (Massagué, 1984) share the same biological properties and potency, and have similar molecular structure. They consist of two apparently identical 12-kDa polypeptide chains linked by disulfide bonds and having a unique primary structure (Derynck et al., 1985). The cellular actions of TGF\(\beta\) are diverse. TGF\(\beta\) was first discovered by its ability to induce certain types of untransformed fibroblasts to grow in semisolid medium (Roberts et al., 1981).

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1 The abbreviations used are: TGF\(\beta\), transforming growth factor-\(\beta\); MEM, Eagle's minimum essential medium; SDS, sodium dodecyl sulfate; NRK, normal rat kidney.

This action of TGF\(\beta\) requires concomitant mitogenic stimulation of the cells with a combination of mitogenic factors (Roberts et al., 1981; Massagué, 1983; Asooian et al., 1984; Massagué et al., 1985). However, fibroblasts which are stimulated to grow in semisolid medium by TGF\(\beta\) can be induced by this factor to prolong their prereplicative period and slow down growth in monolayer culture (Roberts et al., 1985; Shipley et al., 1985). The anchorage-dependent proliferation of epithelial cells and the anchorage-independent growth of certain tumor-derived cell lines are also inhibited by TGF\(\beta\) (Tucker et al., 1984b; Roberts et al., 1985). The observation that TGF\(\beta\) can block adipogenic differentiation without affecting cell growth (Ignozzi and Massagué, 1985) suggests that the primary role of this factor may be to modulate cell development and, as a consequence of that, cell proliferation.

The mechanism mediating these cellular responses to TGF\(\beta\) is not yet known. TGF\(\beta\) binds with high affinity to specific receptors in target cells (Frolik et al., 1984; Tucker et al., 1984a; Massagué and Like, 1985). Information on the structural properties of TGF\(\beta\) receptors has begun to accumulate (Massagué and Like, 1985; Massagué, 1985b). Although stimulation of amino acid and hexose transport and glycolysis by TGF\(\beta\) have been reported (Boerner et al., 1985; Inman and Colowick, 1985), the biochemical events derived from interaction of TGF\(\beta\) with its receptors and responsible for the actions of this factor on cell development remain to be established.

We have examined the possibility that the extracellular matrix is a biochemical target for TGF\(\beta\). The rationale for initiating these studies was twofold. First, the extracellular matrix has a strong influence on cellular functions which are also under the control of TGF\(\beta\), including proliferation, differentiation, and expression of the transformed phenotype (for reviews see Hynes, 1981; Yamada, 1983; Hakomori et al., 1984). Secondly, TGF\(\beta\) in blood platelets is physiologically targeted to intervene in wound healing, a process in which local production and accumulation of extracellular matrix components plays a central role in initiating and directing the compensatory growth of damaged tissue (Kurkinen et al., 1990; Grinnell, 1984). Here we report that treatment of many cell types, normal and transformed, with TGF\(\beta\) increases sharply the levels of fibronectin and collagen, and their incorporation into the matrix. Furthermore, we find that induction of anchorage-independent growth of normal fibroblasts by TGF\(\beta\) can be mimicked with fibronectin and can be inhibited with specific inhibitors of fibronectin action. These findings suggest the possibility that changes in the extracellular matrix mediate the cellular actions of TGF\(\beta\).

EXPERIMENTAL PROCEDURES

Materials—Gelatin-Sepharose and protein A-Sepharose were purchased from Pharmacia-PL Biochemicals. Mouse monoclonal anti-
bodies to cellular chick fibronectin were obtained from Bethesda Research Laboratories and anti-mouse rabbit IgG from Miles Biochemicals. A fluorography enhancer, ENLIGHTNING, [35S]cysteine (1000 Ci/mmol), [3H]leucine (58.4 Ci/mmol), [3H]methyl thymidine (100 Ci/mmol), and [32P]protein A (2-10 Ci/g) were purchased from New England Nuclear. Highly purified, protease-free collagenase was from Worthington. Soybean trypsin and tosylphenylalanyl ketone-treated trypsin were from Sigma. Fibronectins purified by affinity-chromatography from human and chick plasma were from Calbiochem-Behring and Biomedical Technologies (Cambridge, MA), respectively. TGFβ from human placenta (Assay Grade) was purchased labeled with [3H] from DuPont NEN. Preparation of rat anti-mouse rabbit antibodies to cellular chick fibronectin as described before (Massague and Lyle, 1985). Freshly seeded cultures of rat anti-mouse antibody and then subjected to autoradiography.

Collagenase Treatment—The [35S]cysteine-labeled medium was removed from the cultures and centrifuged at 2000 x g for 5 min. The supernatants were retained for further analysis.

Peptides—Synthetic peptides were made in this laboratory using an Applied Biosystems Model 430A Peptide Synthesizer. Peptides were cleaved from the resin and deprotected with hydrogen fluoride in the presence of anisole and methyl ethyl sulfide. The crude peptides were desalted on Sephadex G-10 columns and purified by high performance liquid chromatography on a Vydac C18 column eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid. The purified peptides were lyophilized twice and used in soft agar assays after reconstitution in Dulbecco's MEM under sterile conditions.

Other Assays—For thymidine incorporation assays, chick embryo fibroblasts were grown to confluence in 16-mm wells. The growth medium was replaced with fresh serum-free MEM as the control or supplemented with 5% calf serum, TGFβ, or both. At the indicated times, the test medium was removed and replaced with serum-free MEM containing 2.5 μCi/ml [3H]thymidine. At the end of a 1-h labeling period, the medium was removed and the cells were solubilized in 1% sodium dodecyl sulfate followed by precipitation with 10% trichloroacetic acid. The precipitates were collected by filtration on microporous cellulose ester filters and radioactivity was measured by liquid scintillation counting.

To assay amino acid incorporation into cellular protein, confluent cultures of chick embryo fibroblasts in 10-mm wells were placed into serum-free MEM containing 5% of the normal amount of cysteine or leucine, plus 2.5 μCi/ml [35S]cysteine or 2 μCi/ml [3H]leucine, respectively. Some cultures also received 500 pM TGFβ. Cultures were incubated and allowed to incorporate the radioactive amino acid. At designated times, cultures were harvested by removing the radioactive medium and washing once with cold MEM. Next, the cells were solubilized with 0.5% SDS followed by precipitation in the cold with 10% trichloroacetic acid. The precipitates were collected by filtration on microporous cellulose ester filters and radioactivity was measured by liquid scintillation counting.

Soft agar assays with NRK-49F cells were performed and read as previously described (DeLarco and Todaro, 1978; Massagué, 1983, 1984). Assays were done in duplicate and at least 200 cells and colonies were counted in the readings on day 7.

RESULTS

Increased Fibronectin and Collagen Levels Are a General Response to TGFβ—Cultures of chick embryo fibroblasts synthesized fibronectin as the main component of the extracellular matrix (Yamada and Weston, 1974). We examined the ability of TGFβ to modulate fibronectin levels. After an induction period of 12 h with or without TGFβ, chick embryo fibroblasts were labeled with [35S]cysteine for 3 h as described. Crude urea extracts of the monolayers were made and proteins displayed by dodecyl sulfate-polyacrylamide electrophoresis...
and fluorography. Fig. 1 shows that urea extracts a few high molecular weight proteins which include components of the extracellular matrix as shown by others (Yamada and Weston, 1974). One band of approximately 220 kDa that co-migrated in the gels with purified fibronectin was elevated in the TGFβ-treated cultures, but the level of other proteins was only slightly increased or remained unchanged. To confirm that this 220 kDa band is fibronectin, the urea extracts were subjected to gelatin-Sepharose affinity chromatography (Engvall and Ruoslahti, 1977) or immunoprecipitated with a monoclonal antibody against chick cellular fibronectin. Fibronectin isolated by these two criteria showed the same induction by TGFβ as seen in the urea extracts, a 6–8-fold increase as compared with untreated controls. The figure also shows that greater amounts of labeled fibronectin were also present in the medium from TGFβ-treated cultures as compared to control cultures, although the relative increase of fibronectin in the medium (about 3-fold) was less than in the matrix. When chick cells were grown in the presence of TGFβ, the cultures accumulated greater amounts of fibronectin than cultures not receiving TGFβ as judged by protein immunoblotting (Fig. 1). Thus, the accumulation of a protein identified as fibronectin is increased in cultures of chick embryo fibroblasts treated with TGFβ.

To determine if this is a general function of TGFβ, numerous cell lines were also examined for modulation of the levels of extracellular matrix components (Figs. 2, 3, and 4; Table 1). Urea extracts of various cell lines from human, rat, mouse, and mink origin were subjected to gelatin-Sepharose chromatography for the isolation of fibronectin. Fig. 2 shows some representative cell lines and the induction of fibronectin by TGFβ. Hence, the induction of fibronectin by TGFβ appears to be a general phenomenon in fibroblastic and epithelial cell lines. TGFβ induction of fibronectin occurs even in cells which normally produce very little fibronectin such as NRK fibroblasts.

Some of the cell lines examined do not synthesize fibronectin as the major extracellular matrix protein. In these lines proteins of 140, 170, and/or 190 kDa were elevated following TGFβ stimulation (Fig. 3). The level of a 250 kDa matrix protein unique to WI-38 cells (Carter, 1982) was also elevated by TGFβ (Fig. 3). Like fibronectin, the 140–190 kDa proteins accumulated in the conditioned medium from TGFβ-treated cells.

**Fig. 1.** Effect of TGFβ on extracellular matrix components from chick embryo fibroblasts. Twenty-four hours after seeding, confluent monolayers of secondary chick embryo fibroblasts were transferred to serum-free medium with (+) or without (−) 0.5 nM TGFβ. After 12 h, the medium was replaced with cysteine-free medium containing [35S]cysteine with or without TGFβ. After 3 h, the medium was collected and monolayers were extracted in the cold with buffer containing 1 M urea. Aliquots of the solubilized material diluted with an equal volume of 1% Triton X-100 and aliquots of culture medium were incubated for 18 h with 50 µl of packed gelatin-Sepharose beads, and the bound material was eluted with SDS-sample buffer. Other aliquots diluted and made to 0.5% Triton X-100 were incubated for 18 h at 4 °C with anti-chick fibronectin monoclonal antibody, and the immunocomplexes precipitated with rabbit anti-mouse IgG and protein A-Sepharose. The urea extracts from the extracellular matrix, the eluates from the gelatin-Sepharose beads and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis on 5% gels and fluorography (24 h). In a separate experiment, monolayers incubated for 12 h in serum-free medium with (+) or without (−) 0.5 nM TGFβ were rinsed, extracted with 1 M urea, and subjected to SDS-polyacrylamide gel electrophoresis, followed by electroblot transfer onto a nitrocellulose filter for 18 h. The filter was sequentially incubated with anti-chick fibronectin antibody, anti-mouse IgG, and [125I]-protein A, and subjected to autoradiography (3 days). Shown are the fluorograms and autoradiograms corresponding to these experiments. Arrow shows the position of purified chick fibronectin migrating in the same gels and detected by protein staining or immunoblotting. Numbers indicate the position and molecular mass (kDa) of protein standards in the gels.

**Fig. 2.** Increased labeling of fibronectins seen in various cell lines. Confluent cultures of human WI-38, rat NRK, mouse BALB/c 3T3, and mink Mv1Lu cells were treated with or without TGFβ, labeled with [35S]cysteine, and extracted with a urea-containing buffer as in Fig. 1. The extracts were subjected to gelatin-Sepharose affinity chromatography as described and the isolated fibronectin was subjected to gel electrophoresis and fluorography. FN, fibronectin.

**Fig. 3.** Effect of TGFβ on the extracellular matrix is a general phenomenon. Approximately 36 h after plating, the cultures were transferred to serum-free MEM with (+) or without (−) 500 µM TGFβ for 12 h. The medium was then replaced with serum-free, cysteine-free MEM containing 20 µCi/ml [35S]cysteine and TGFβ as before. After a 3-h labeling period, the medium was removed, and cultures were washed and extracted with urea-containing buffer. The extracted proteins were electrophoresed on 5% polyacrylamide-dodecyl sulfate gels and subjected to fluorography. Equal counts/min (~2.5 × 10⁶) were loaded from control and TGFβ-treated cultures. Numbers and dots indicate the position of molecular mass markers in kilodaltons. Arrowheads, TGFβ-induced proteins.
cells (Fig. 4). The 140–190 kDa proteins whose labeling was elevated following TGFβ treatment are likely to be procollagens and related species. Thus, human WI-38, rat NRK, and mouse 3T3 cells are known to synthesize and incorporate into the matrix higher amounts of collagen-like proteins of 140, 170, and 190 kDa than fibronectin (Carter, 1982; Hakomori et al., 1984). The 170 and 190 kDa proteins have been identified as pro-α1 and pro-α2 procollagens, respectively (Krieg et al., 1980; Carter, 1982). Fig. 5 shows that the 140, 170, and 190 kDa proteins from mouse 3T3-L1 cells were selectively digested by collagenase. Other proteins in the gels were not altered by collagenase treatment (Fig. 5). Similar results were obtained with mouse BALB/c 3T3 cells, human WI-38 cells and rat NRK and L6E9 cells (not shown).

The results of experiments with various cell lines are compiled in Table I. TGFβ modulates the synthesis of fibronectin and 140-190 kDa (collagen-related) matrix proteins in a variety of cell lines. These cell types include NRK-49F and BALB/c 3T3 fibroblasts which are stimulated to proliferate in soft agar by TGFβ (DeLarco and Todaro, 1978; Massagué et al., 1985), SV40-transformed WI-38 fibroblasts (WI-38 VA-13) and tumor-derived A549 cells which are growth-inhibited by TGFβ in soft agar (Roberts et al., 1985; Iwata et al., 1985), Mv1Lu epithelial cells whose proliferation in monolayer cul-

![Fig. 4. Matrix proteins soluble in the culture medium also show increased labeling following TGFβ treatment. Cultures of various cell lines were treated with or without 500 pm TGFβ as described in the legend to Fig. 3. After the labeling period, the medium was removed and clarified by centrifugation at 2000 × g after addition of 1 mM phenylmethylsulfonyl fluoride. Equal volumes of medium from control and treated cultures were analyzed by gel electrophoresis and fluorography. Arrowheads, TGFβ-induced proteins.](image)

![Fig. 5. Some of the labeled proteins are collagen-like molecules. Confluent cultures of 3T3-L1 cells were treated with or without 500 pm TGFβ for 9 h, followed by a 3-h labeling period as before. The conditioned culture medium was collected and treated as in Fig. 4. Samples were digested with collagenase (100 units/ml) for 12 h at 22 °C. Undigested samples were treated exactly the same but without the addition of collagenase. Samples were analyzed by gel electrophoresis and fluorography. Collagenase-sensitive proteins of 140, 170, and 190 kDa are labeled using Carter’s nomenclature (Carter, 1982). FN, fibronectin.](image)

**TABLE I**

Effect of TGFβ on extracellular matrix proteins in various cell lines

Near-confluent cultures were incubated for 12 h with 0.5 nM TGFβ and labeled for 3 h with [35S]cysteine. Matrix proteins were extracted with urea-containing buffer and displayed by SDS-PAGE and fluorography. Individual bands were quantitated by scanning densitometry and/or counting the radioactivity in the corresponding gel slices. The results are expressed as fold induction of the corresponding protein relative to controls that did not receive TGFβ. FN, fibronectin.

<table>
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<td>HOS</td>
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<tr>
<td>CEF</td>
<td>Chicken embryonic fibroblast</td>
<td>6.00</td>
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† Samples of the labeled conditioned medium rather than cell extracts were analyzed.

* Indicates no effect.
tis is inhibited by TGFβ (Tucker et al., 1984b), and 3T3-L1 preadipocytes and various lines of myoblasts whose differentiation is regulated by TGFβ (Ignotz and Massagué, 1985).2

Characterization of Chick Embryo Fibroblasts as a Model System for TGFβ Action—To further characterize the actions of TGFβ we chose chick embryo fibroblasts and fibronectin synthesis as a model system. This choice was based on the marked responsiveness of fibronectin levels in these cells to TGFβ, and on the fact that this is one of the best characterized systems in terms of fibronectin structure and expression. TGFβ has diverse effects on cell proliferation, in some instances acting as a stimulator of cell growth and in others as an inhibitor (Tucker et al., 1984b; Roberts et al., 1985). We determined what type of growth response TGFβ does elicit in chick embryo fibroblasts. TGFβ was found to prolong the prereplicative period of chick embryo fibroblasts stimulated with serum (Fig. 6). These results are similar to those obtained with mouse AKR-2B fibroblasts (Shipley et al., 1985).

Selectivity and Specificity of TGFβ Effect on Fibronectin Levels—TGFβ induced an average 34% increase in overall protein synthesis in chick embryo fibroblasts by 6 – 12 h, as measured by [35S]cysteine or [3H]leucine incorporation into trichloroacetic acid-precipitable cellular material (not shown). This increase in cellular protein is in contrast to the increase in cellular fibronectin induced by TGFβ, which is 300 – 400% at 6 h (see below). When equivalent amounts of cellular extracts were analyzed by gel electrophoresis, the pattern seen reflected the general increase in protein synthesis described above. A slight increase was observed in nearly all the bands following TGFβ stimulation (Fig. 7). However, a band of 220 kDa presumed to be fibronectin showed a more marked increase in labeling.

Other growth factors were individually tested for the ability to affect fibronectin production in chick embryo fibroblasts. Fig. 8 shows that epidermal growth factor and fibroblast growth factor are ineffective in increasing labeling of fibronectin in the extracellular matrix. Insulin-like growth factor-II was reproducibly found to induce an increase in fibronectin. However, in contrast to TGFβ, insulin-like growth factor-II increased overall protein synthesis 2.2-fold 15 h after addition to the cells (not shown), as expected from its strong mitogenic activity on chick embryo fibroblasts (Dulak and Temin, 1973).

Kinetics of Elevation of Intracellular and Matrix Fibronectin by TGFβ—We examined the kinetics and relative change of intracellular fibronectin in parallel with fibronectin in the extracellular matrix and in the medium. Mild digestion of cultures with trypsin removes the matrix fibronectin but leaves intracellular fibronectin (Olden and Yamada, 1977). Fig. 9 shows that intracellular fibronectin production was approximately 3-fold higher in the TGFβ-stimulated cells.
serum-free medium containing no additions the factors. Following a 3-h labeling period, the cultures were extracted with the urea-containing buffer and the extracts subjected to gelatin-Sepharose affinity chromatography. The isolated fibronectin factor persisted at this level until at least 24 h after TGFβ addition (not shown). These results suggest that the effect of TGFβ on fibronectin in the matrix is in part secondary to an increase in the intracellular levels of fibronectin. Fig. 9 also shows that the kinetics of accumulation of fibronectin in the medium were similar to those in the matrix. However, the relative increase of fibronectin in the matrix was much higher than in any of the other two compartments, suggesting that cells treated with TGFβ may also have a higher binding capacity for fibronectin.

Fig. 10 shows the dose-response relationship of the effect of TGFβ on extracellular matrix fibronectin. A half-maximal effect was obtained with 100–250 pm TGFβ when the measurements were done at 12 h. Control experiments using tracer amounts of 125I-TGFβ indicated that at this time point 40, 37, or 34% of the added TGFβ had been degraded by the monolayers when 50, 250, or 500 pm concentrations of the factor were used, respectively. Thus, the half-maximal effect of TGFβ is estimated to occur at about 100 pm concentration.

Transcription Involved in the Response—The results are consistent with an induction of fibronectin expression by TGFβ. To further examine this possibility, cultures were treated with actinomycin D simultaneously with TGFβ. Actinomycin D blocked the increase in fibronectin levels by TGFβ but did not decrease the levels of fibronectin in cells not treated with TGFβ (Fig. 11). This suggests that transcription of RNA, presumably fibronectin mRNA, is required for the increased fibronectin protein production.

A Functional Role of Fibronectin in TGFβ Action—The results described above raised the possibility that TGFβ may influence cell growth and development by regulating the abundance and composition of the extracellular matrix. We tested the ability of purified fibronectin to mimic cellular actions of TGFβ. Induction of proliferation of NRK-49F fibroblasts in semisolid medium is the first and most widely studied cellular action of TGFβ (DeLarco and Todaro, 1978; Roberts et al., 1981). We find that fibronectin from human or chick plasma (Fig. 12A) can substitute for TGFβ to induce proliferation of NRK-49F cells into tightly packed spherical colonies in soft agar medium. Like TGFβ, fibronectin action requires the concomitant stimulation of cells with epidermal growth factor plus other mitogens provided by serum.

We have used the synthetic hexapeptide Gly-Arg-Gly-Asp-Ser-Pro, a competitive inhibitor of fibronectin binding to cell surfaces (Pierschbacher and Ruoslahti, 1984a) to further assess the involvement of the extracellular matrix in the action
Extracellular Matrix Control by TGFβ

**FIG. 10. Effect of TGFβ dose on fibronectin accumulation in chick embryo fibroblasts.** Confluent cultures of chick embryo fibroblasts were placed in serum-free MEM supplemented with the indicated concentrations of TGFβ. After 12 h, the cultures were labeled with [35S]cysteine and extracted with the urea-containing buffer as before. Fibronectin was isolated by gelatin-Sepharose affinity chromatography and subjected to gel electrophoresis. Shown is the resulting fluorogram and the change in fibronectin content at the indicated concentrations of TGFβ after normalization to control cultures not receiving TGFβ. Quantitation of labeled fibronectin was done as in Fig. 9.

**FIG. 11. Effect of actinomycin D on fibronectin production.** Cultures of chick embryo fibroblasts were treated with actinomycin D at the indicated concentrations, with or without 500 pM TGFβ for 12 h, followed by a 3-h labeling period as before. The labeling medium contained the same additions as prior to labeling. Urea extracts of the monolayers were made and subjected to gelatin-Sepharose affinity chromatography. Isolated fibronectin was analyzed by gel electrophoresis and fluorography.

**FIG. 12. Involvement of extracellular matrix proteins and their cellular receptors in soft agar growth of normal fibroblasts in response to TGFβ.** NRK-49F cells were plated in Dulbecco's MEM containing 10% calf serum and 0.5 nM epidermal growth factor plus (A) the indicated concentrations of fibronectin from human plasma (○) or chick plasma (●); or (B) 50 pM TGFβ and the indicated concentrations of the synthetic peptides, Gly-Arg-Gly-Asp-Ser-Pro (●) and Gly-Arg-Gly-Glu-Ser-Pro (○). ▲ in B indicates the control value obtained with calf serum plus epidermal growth factor only. Assays were read on day 7. Results are expressed as the percent of cells that formed colonies. Assays were done in duplicate; all experiments were repeated at least once and gave similar results. TGFβ (50 pM) assayed in control dishes in the two experiments shown in A gave 49 and 72% colonies, respectively.

of TGFβ on NRK-49F cells. This peptide mimics the site in the fibronectin molecule that binds to specific cell surface receptors. Short peptides containing the Arg-Gly-Asp-Ser sequence can inhibit fibronectin binding to cells, prevent attachment of cells to fibronectin-coated surfaces, and elute the putative fibronectin receptor from immobilized fibronectin columns (Pierschbacher and Ruoslahti, 1984a; Pytela et al., 1985). Substitution of a glutamic acid for the aspartic acid residue renders this sequence inactive (Pierschbacher and Ruoslahti, 1984b). Gly-Arg-Gly-Asp-Ser-Pro was found to inhibit in a concentration-dependent manner the induction of NRK-49F cell colonies by TGFβ in semisolid medium (Fig. 12B). In the same experiments, the hexapeptide Gly-Arg-Gly-Glu-Ser-Pro was without effect (Fig. 12B). These results suggest that fibronectin presumably derived from stimulation of NRK-49F cells with TGFβ facilitates the formation of a local matrix that allows proliferation of the cells in suspension.

**DISCUSSION**

Numerous reports during recent years have shown that changes in the composition of the extracellular matrix have profound effects on cell proliferation and differentiation. The development of many cell types in vitro can be manipulated by altering the levels of fibronectin, collagens, and other major components of the matrix. Another widely reported phenomenon is the association of oncogenic transformation with abnormalities in the expression of fibronectin (Hynes, 1973; Stone et al., 1974; Vaheri and Ruoslahti, 1974), collagens (Green et al., 1966; Levinson et al., 1975; Hata and Peterkofsky, 1977), and other matrix components (Terranova et al.,...
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In addition, physiologically normal processes involving acute cell growth and differentiation, such as wound healing, are associated with complex patterns of extracellular matrix accumulation (Grinnell, 1984). The remarkable parallelism that exists between the types of cellular responses elicited by TGFβ and the effects of the extracellular matrix on cell development led us to consider the possibility that a key event in the action of TGFβ could be the induction of changes in the composition and/or abundance of extracellular matrices. The presence of TGFβ in many tissues, and the role of platelets as a major reservoir of this factor also made the hypothesis plausible.

The results reported here demonstrate that the induction of fibronectin and collagen-related proteins and their incorporation into the extracellular matrix is a general response of cells to TGFβ. As a consequence of this effect, the relative proportion of newly synthesized fibronectin and collagen levels in the matrix changes. These observations suggest that, in addition to an increase in matrix material, there is a change in the composition of the extracellular matrix in response to TGFβ. Using fibroblast and chick embryo fibroblasts as a model system for a more detailed study of the effect of TGFβ, we find that the increase in fibronectin is an early event in the action of TGFβ and persists for many hours thereafter. The effect of TGFβ on fibronectin production is selective as compared with the modest effects on overall protein synthesis and increases the ability of chick fibroblasts to incorporate fibronectin levels via an alternative mechanism.

The results indicate that the effect of TGFβ is based at least in part on an increase in the production of fibronectin. Thus, the increase in the rate of fibronectin production in TGFβ-treated chick fibroblasts is observed in all cellular compartments, including intracellular, matrix, and medium. Kinetically, the effect on intracellular fibronectin precedes the effect on extracellular fibronectin. The ability of actinomycin D to block the effect of TGFβ is also compatible with an increased synthesis of fibronectin. It remains to be determined whether TGFβ stimulates directly actinomycin D-sensitive transcription of the fibronectin gene or increases fibronectin levels via an alternative mechanism.

In addition to stimulating fibronectin production, TGFβ increases the ability of chick fibroblasts to incorporate fibronectin into the matrix. The relative increase in extracellular matrix fibronectin levels in TGFβ-treated cells is much higher than the increase of fibronectin in the medium or inside the cells. Changes in the cells' ability to incorporate fibronectin have also been observed in some transformed cells which produce close to normal levels of fibronectin but fail to incorporate it (Vaheri and Ruoslahti, 1975; Wagner et al., 1981). The possibility that the cellular receptor for fibronectin (Pytel et al., 1985; Hasegawa et al., 1985) is up-regulated by TGFβ deserves further attention.

Collagen-related proteins are the major components of the extracellular matrix in many of the cell lines examined. The identification of the 140, 170 kDa (pro-a2 procollagen), and 190 kDa (pro-a2 procollagen) as collagen-related proteins is based on their sensitivity to digestion by collagenase, and on previous reports characterizing in detail matrix proteins with the same molecular properties as those observed here (Carter and Hakomori, 1981; Carter, 1982). Fibronectin contains a binding site for collagen (Balian et al., 1979; Ruoslahti et al., 1979; Hahn and Yamada, 1979). However, the increase in 140, 170, and 190 kDa proteins in the extracellular matrix of TGFβ-treated cells is not merely due to the increased binding capacity of an elevated fibronectin content in the matrix. The increase in 140, 170, and 190 kDa proteins occurs in the medium as well as in the matrix and is seen even in cells that produce very little fibronectin. It is likely that TGFβ elicits a pleiotropic action similar but opposite to the action of transformation on the extracellular matrix components. Previous reports have indicated that the concomitant decrease in the expression of fibronectin and collagen in transformed cells is caused by common overlapping mechanisms. Transformation decreases the transcriptional activity of fibronectin and collagen genes (Howard et al., 1979; Sandmeyer et al., 1981; Tyagi et al., 1983), an action that may involve trans-acting factors regulating the expression of these genes (Setoyama et al., 1985). It is possible that the same mechanism is under the control of TGFβ.

The changes in abundance and composition of extracellular matrices induced by TGFβ can clearly have profound effects on cell development. Effects of TGFβ on the extracellular matrix may mediate simultaneously opposite cellular actions of this factor. For example, induction of anchorage-independent growth of NRK-49F and BALB/c 3T3 fibroblasts by TGFβ (DeLaRoc and Todaro, 1978; Roberts et al., 1981; Massagué et al., 1985) could be mediated by the accumulation of an abundant extracellular matrix surrounding the cells and providing enough anchoring support for growth even in semisolid medium. Two predictions that can be made from this hypothesis are that the promotion of NRK-49F cell proliferation in soft agar medium by TGFβ may be mimicked by fibronectin and should be inhibited by inhibitors of fibronectin binding to its cell surface receptor; the results reported here confirm both predictions. Therefore, growth of NRK-49F cells in soft agar induced by TGFβ is not part of a typical transformed phenotype as previously thought. In contrast, SV40-transformed human WI-38 fibroblasts, which are shown here to increase fibronectin and collagen levels in the presence of TGFβ, respond to TGFβ (Iwata et al., 1985) as well as to direct addition of fibronectin (Yamada et al., 1979) with a partial normalization of the transformed phenotype. Similar observations have been made with A549 human lung carcinoma cells (Roberts et al., 1985).

The regulatory effects on extracellular matrix components described here shall help understand the physiological role of TGFβ and the basis for its cellular actions while providing for the first time a system to address the nature of post-receptor events elicited by this factor.

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