

Transforming Growth Factor- β Stimulates the Expression of Fibronectin by Human Keratinocytes

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Transforming growth factor beta (TGF- β) is a 25-kD protein which has regulatory activity over a variety of cell types. It is distinct from epidermal growth factor (EGF) and EGF analogs, and exerts its action via a distinct receptor. Its effect on proliferation or differentiation can be positive or negative depending on the cell type and the presence of other growth factors. It also modulates the expression of cellular products. TGF- β causes fibroblasts to increase their production of the extracellular matrix components, fibronectin and collagen. Human keratinocytes (HK) are known to have TGF- β receptors. We wished to study the effect of TGF- β on the production of extracellular matrix proteins by human keratinocytes in culture.

Human keratinocytes were grown in serum-free defined medium (MCDB-153) to about 70% confluence. Following

a 16-h incubation in medium lacking EGF and TGF- β , cells were incubated for 12 h in medium containing varying concentrations of EGF and TGF- β . Cells were then labeled with ^{35}S -methionine for 10 h in the same conditions. Labeled proteins from the medium were analyzed by SDS-PAGE and autoradiography.

TGF- β at 10 ng/ml induced a sixfold increase in the secretion of fibronectin, as well as an unidentified 50-kD protein. Thrombospondin production was also increased, but not over a generalized twofold increase in the production of all other proteins. EGF, at 10 ng/ml, caused a smaller additive effect. TGF- β may be an important stimulator of extracellular matrix production by human keratinocytes. *J Invest Dermatol* 91:207-212, 1988

Transforming growth factor- β (TGF- β) is a 25-kilodalton (kD) protein originally described in 1981 [1] and named for its ability, in the presence of other growth factors, to promote the attachment-independent proliferation of certain fibroblastic cell lines in soft agar.

Since this description, TGF- β has been found to have diverse actions on a wide variety of cell types (for a recent review see Ref 2).

The effects of TGF- β depend on the cell type under study. Thus TGF- β inhibits the proliferation and/or differentiation of certain fibroblast [3] and epithelial cell lines [4-6]. Depending on the system under study, the effects of TGF- β may depend upon, be modified by, or antagonize the effects of other growth factors.

Ignatz and Massagué [7] have demonstrated that TGF- β stimulates the synthesis and deposition into the extracellular matrix of fibronectin and collagen by fibroblasts and myoblasts. They also have shown stimulation of fibronectin (but not collagen) secretion by two transformed epithelial cell lines. Because untransformed human keratinocytes have been shown to display functional receptors to TGF- β [5], it was of interest to examine the effect of TGF- β on extracellular matrix production by human keratinocytes.

Epidermal growth factor (EGF) is a potent growth factor for human keratinocytes [8]. In addition, several interactions between the actions of TGF- β and EGF have been reported. Thus TGF- β has been shown to inhibit the clonal growth of human keratinocytes, even in the presence of EGF [5]. At the receptor level TGF- β has been reported to increase the binding of EGF to NRK fibroblasts under certain conditions [9], while reducing the number of high-affinity EGF receptors under other conditions [10]. Other studies have shown inhibitory effects of TGF- β on EGF function at levels distal from the EGF receptor [11,12]. Because of these interactions between the effects of TGF- β and EGF, we also wished to examine the effect of combinations of TGF- β and EGF on extracellular matrix production.

MATERIALS AND METHODS

Materials Except as noted, all chemicals were from Sigma (St. Louis, MO). TGF- β , isolated from bovine bone [13], was the generous gift of John McPherson (Connective Tissue Research Laboratories, Collagen Corporation, Palo Alto, CA). EGF was from Collaborative Research, Lexington, MA.

Keratinocyte Culture Pure human keratinocyte cultures were derived from human neonatal foreskins. They were grown in serum-free medium (MCDB 153) according to the method of Boyce and Ham [14] with modifications as described previously

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Abbreviations:

- BPE: bovine pituitary extract
- EGF: epidermal growth factor
- Fn: fibronectin
- kD: kilodalton
- NEM: N-ethyl maleimide
- PAS: protein A Sepharose
- PASW: PAS wash
- PASW-OVA: PAS wash with 0.1% ovalbumin
- PBS: phosphate-buffered saline
- PMSF: phenylmethylsulfonyl fluoride
- TGF- β : transforming growth factor- β
- TSP: thrombospondin
- SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

[15]. The final medium contained 0.1 mM calcium, 10 $\mu\text{g}/\text{ml}$ EGF. Cells grown in such media exhibited a basaloid phenotype and did not cornify or stratify, but continued to proliferate, as has been described [14,16]. Keratinocytes were used at second or third passage. The use of such passaged cells resulted in the virtual elimination of all contaminating cell types [15] as well as better repeatability in numbers of cells plated. Bovine pituitary extract was used in bringing up the primary cell cultures, and in growing up the cells to final density in the experimental plates or slides. It was then eliminated as described below. For metabolic labeling studies, human keratinocytes were grown on 35 mm plastic dishes (Falcon, Oxnard, California) to 70% to 80% confluence. Sixteen hours prior to placing the cells in experimental conditions, the cells were washed with phosphate buffered saline (PBS) and placed in MCDB 153 without bovine pituitary extract (BPE) or in MCDB 153 without BPE and without EGF. For immunofluorescence studies human keratinocytes were grown in glass LabTek chamber slides (Miles Laboratories, Naperville, IL).

Experimental Conditions At time zero ($t = 0$ h) the cells were washed and placed in experimental conditions (2 ml/35 mm dish or 1 ml/slide chamber). These consisted of MCDB without BPE and containing varying combinations of TGF- β and EGF as described in "Results." In radiolabeling studies, at $t = 12$ h, cells were placed in 1.6 ml/plate of media containing the same concentrations of TGF- β and EGF and also 25 $\mu\text{Ci}/\text{ml}$ of ^{35}S -methionine (ICN Radiochemicals, Irvine, CA). This preparation also contains ^{35}S -cysteine, such that there was a total of approximately 15 $\mu\text{Ci}/\text{ml}$ in the experimental conditions. All samples were run at least in duplicate. For immunofluorescence studies cells were kept in experimental conditions for 72 h, with a change of media at 48 h.

Detection of Secreted Proteins At $t = 22$ h, labeled conditioned media were removed from the plates. 100x stocks of phenylmethylsulfonyl fluoride (PMSF) (Calbiochem-Behring, La Jolla, California), *N*-ethyl maleimide (NEM) (Calbiochem), pepstatin (in ethanol), and EDTA (pH 7.4 in water) were added to the media to final concentrations of 1 mM, 5 mM, 5 $\mu\text{g}/\text{ml}$, and 5 mM, respectively. Media were centrifuged at 10,000 $\times g$ for 20 sec to remove any cellular debris, then dialyzed against 4 changes of 5 mM ammonium acetate containing 1 mM PMSF, and then one change of 1 mM ammonium acetate (volume ratio: 50:1, 2 h/change, 4°C). The dialyzed media were freeze-dried, then resuspended in 100 μl of Laemmli [17] sample buffer containing 2-mercaptoethanol. Samples were run on sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) and autoradiography was performed as previously described [15] except that 5% to 15% gradient gels were used. Quantitation of various bands on the autoradiographs was obtained via two methods: 1) scanning densitometry was performed on a Zeineh Model SL-2D Scanning Laser Densometer (Bio Med Instruments, Fullerton, CA) or 2) the relevant bands were cut from the dried gels using the autoradiographs as a guide; these bands were then solubilized in H_2O_2 at 70°C, suspended in Scinti VerseTM II (Fisher Scientific Co., Fair Lawn, NJ), according to the method of Richies [18], and counted on a Beckman (Beckman Instruments, Inc., Irvine, CA) LS 1801 beta counter. Aliquots of the resuspended dialyzed media were similarly counted as a measure of total protein secretion.

Pepsin and Collagenase Digestion Samples for pepsin digestion were treated as above, except that no enzyme inhibitors were added; the pH of the sample was lowered to about 2 by the addition of a 1/100th volume of 10 N HCl; pepsin, 5 mg/ml, was added to a final concentration of 50 $\mu\text{g}/\text{ml}$; and the samples were incubated overnight with gentle rocking at 4°C. The reaction was halted by the restoration of the pH to approximately 7 by the addition of 1/100th volume of 10 N NaOH and by the addition of pepstatin to 5 $\mu\text{g}/\text{ml}$ as above. Samples for collagenase digestion were treated as above, except that no EDTA was added, NEM and PMSF were added to final concentrations of 2.5 mM and 100 nM, respectively, calcium chloride (0.5 M) was added to a final concentration of 5 mM, and collagenase ("Form III", Advance Biofactures Corp.,

Lynbrook, NY) was added to a final concentration of 150 units/ml. The samples were incubated for 2 h at 37°C, then overnight at room temperature. The reaction was stopped by the addition of 0.5 M EDTA to a final concentration of 5 mM.

All digested samples were then dialyzed and freeze dried as above.

Immunoprecipitation Immunoprecipitation of fibronectin was performed according to the method described [15] employing a rabbit anti-fibronectin antibody [19] and protein A Sepharose 4B (PAS) (Pharmacia). Briefly, 1 ml samples of labeled medium were pre-cleared for 2 h at 4°C with 100 μl of a 20% slurry of PAS in PAS wash (PASW) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM cold methionine, 0.5% NP-40, 0.02% sodium azide) containing 0.1% ovalbumin (PASW-OVA). After centrifugation, samples were incubated overnight at 4°C with 10 μl of antibody (at approximately 1 mg/ml). Next 100 μl of 20% PAS in PASW-OVA was added to the samples. After 2 h incubation with gentle rocking at 4°C, the beads were washed five times with PASW-OVA, once with PASW, and once with 50 mM Tris pH 7.4. 50 μl of Laemmli [17] sample buffer containing 2-mercaptoethanol was added to the pellets. These were boiled for 5 min, spun, and the supernatants applied directly to the lanes of polyacrylamide gels (3% stacking gel, pH 6.8, 5%–15% gradient separating gel, pH 8.8) and run under reducing conditions. Gels were dried and autoradiographed without intensifying screens.

Immunofluorescence Cells were fixed for 15 min at 37°C in 2% formaldehyde (made fresh from paraformaldehyde) and 1% NP-40 in PBS. Staining was performed using an avidin-biotin technique as previously described [20] using a sheep anti-human fibronectin as a primary antibody. Stained slides were photographed using an Olympus (Olympus Corporation, Lake Success, NY) VANOX-T Model AH-2 microscope equipped for UV epifluorescence. A rough quantitation of the degree of IF staining was obtained by using the microscope's photometer on a field-averaging setting, and recording the photometric readings for 20 consecutive randomly selected low power (10x) fields. The intensity of staining was taken as the reciprocal of this number.

Analysis of Data Differences between the quantities of various secreted proteins under various combinations of TGF- β and EGF were analyzed with two-way analysis of variance. In calculations on fold increases, standard errors of these fold increases (standard errors of ratios of means) were computed using bootstrap resampling of 1000 synthetic samples per ratio [21]. The difference between immunofluorescence staining with or without TGF- β was analyzed with Student's *t* test. Standard statistical calculations were performed using "Stat-Works" (Cricket Software, Philadelphia, PA) on a Macintosh Plus Computer (Apple Computer, Cupertino, CA). The bootstrap calculations were performed using a program written by one of the authors (NEW).

RESULTS

As an initial test of the effects of EGF and TGF- β , cell cultures were incubated in the absence of bovine pituitary extract, but in the presence of EGF at 10 ng/ml for 16 h prior to being placed in experimental conditions. Next the culture plates were washed and switched into experimental conditions for 22 h, the final 10 of which were with radioactive label. These conditions consisted of all nine combinations of concentrations of EGF of 0, 3, and 10 ng/ml and concentrations of TGF- β of 0, 3, and 10 ng/ml. Each condition was run in duplicate. Collection and analysis by SDS-PAGE and autoradiography was performed as described in "Materials and Methods." Visual inspection of the data and two-way analysis of variance showed no effects of varying concentrations of EGF (data not shown). This allowed the data for all EGF concentrations to be combined, yielding six replicates for each TGF- β concentration and a more precise determination of the dose-response for TGF- β . Variations in the TGF- β concentration from 0 to 10 ng/ml produced striking differences both in the overall amount of protein synthesis and in the relative quantities of several major proteins.

Figure 1 shows autoradiographs of three representative lanes from this experiment. The lanes represent the pattern of metabolically labeled secreted proteins produced by human keratinocytes in conditions of 0, 3, and 10 ng/ml of TGF- β . The dark band at approximately 220 kD has been identified as fibronectin [20]. The identification of this band as fibronectin was reconfirmed by immunoprecipitation (Fig 2). The second dark band at 180 kD has been shown to be thrombospondin [15]. These are the most prominent labeled secreted proteins at [TGF- β] of zero. At TGF- β concentrations of 3 and 10 ng/ml, however, several changes are seen. First, there is a marked increase in overall secretion of metabolically labeled proteins. Second, several new bands appear. Most prominent among these is a band migrating at an apparent molecular weight of 50 kD. This 50-kD band, as well as the fibronectin and thrombospondin bands, were selected for further analysis by densitometry.

The overall increase in secretion of labeled proteins between [TGF- β] of zero and [TGF- β] of 10 ng/ml was threefold (data not shown). The *n*-fold increases for fibronectin, thrombospondin, the 50-kD protein, and all other proteins, as determined by densitometry, are shown graphically in Fig 3. The 50-kD protein underwent a 6.3-fold increase and fibronectin underwent a 5.5-fold increase, while thrombospondin and all other proteins underwent twofold and 2.8-fold increases, respectively. These increases were nearly maximal at [TGF- β] of 3 ng/ml. Analysis of variance showed all of these increases to be highly significant, whether expressed as absolute increases, or as fractions of total protein secretion. *P* values for the absolute increases in fibronectin, thrombospondin, and the 50-kD protein were <0.001, 0.003, and <0.001, respectively.

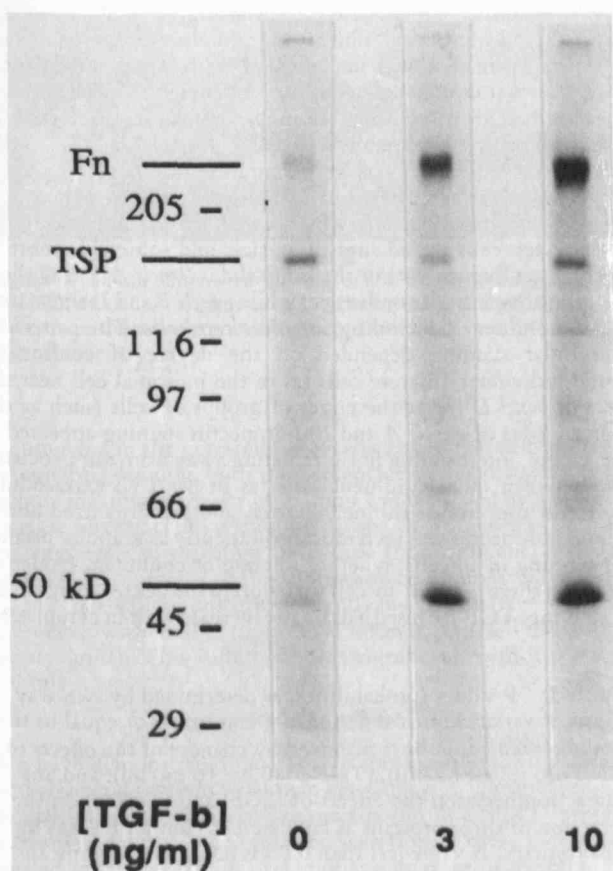


Figure 1. Autoradiograph of SDS-PAGE gels of labeled proteins obtained from conditioned medium from human keratinocytes incubated in TGF- β concentrations of 0, 3, and 10 ng/ml, as indicated at the bottom of each lane. The migration positions of molecular weight markers, as well as the positions of fibronectin (*Fn*), thrombospondin (*TSP*), and a 50-kD protein are shown on the left.

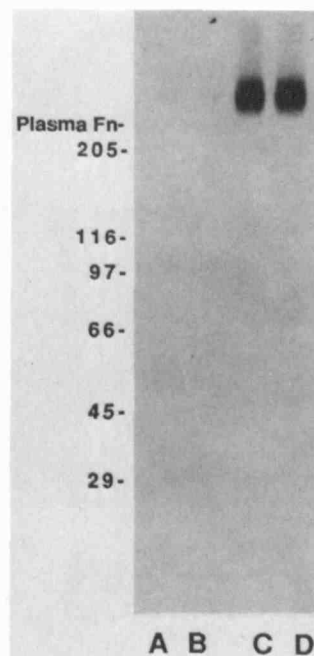


Figure 2. Autoradiograph of immunoprecipitates using rabbit anti-fibronectin as antibody. Immunoprecipitates of labeled proteins from conditioned media of duplicate culture dishes are shown. Lanes A and B are from unstimulated human keratinocyte cultures; Lanes C and D are from human keratinocyte cultures stimulated with 10 ng/ml of TGF- β . The migration positions of plasma fibronectin (Plasma Fn) and molecular weight markers are shown on the left.

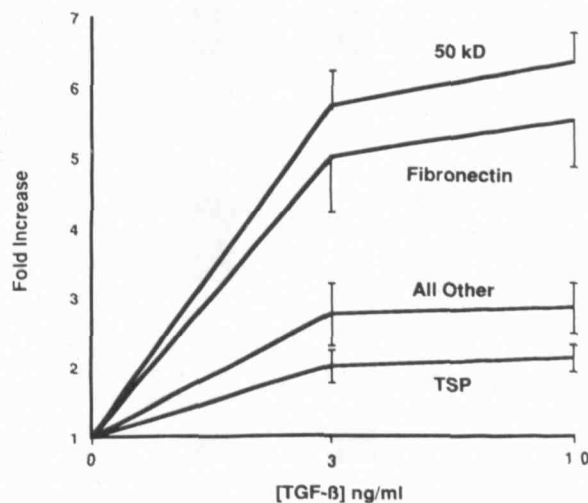


Figure 3. Fold increases (values obtained from TGF- β -stimulated human keratinocyte conditioned medium over the values obtained from unstimulated human keratinocyte conditioned medium) of thrombospondin (*TSP*), fibronectin, and the 50-kD protein, as well as the average of all other proteins (as indicated near each line), are plotted as a function of [TGF- β]. Each point represents the average amount of the particular protein detected from six replicate dishes in the indicated condition divided by the average amount of the particular protein detected from six replicate dishes of unstimulated cells. Error bars are the standard errors of ratios of means of six items, as determined by a bootstrap resampling technique (see "Methods").

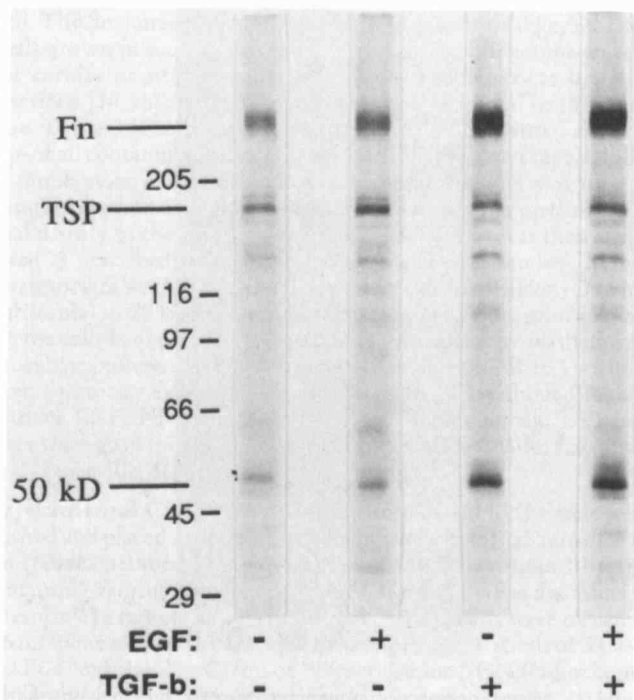


Figure 4. Autoradiograph of SDS-PAGE gels of labeled proteins obtained from conditioned medium from human keratinocytes incubated in four combinations of concentrations of EGF and TGF beta as indicated below the lanes. Migration positions of molecular weight markers and band identifications (Fn = fibronectin, TSP = thrombospondin) are as shown on the left.

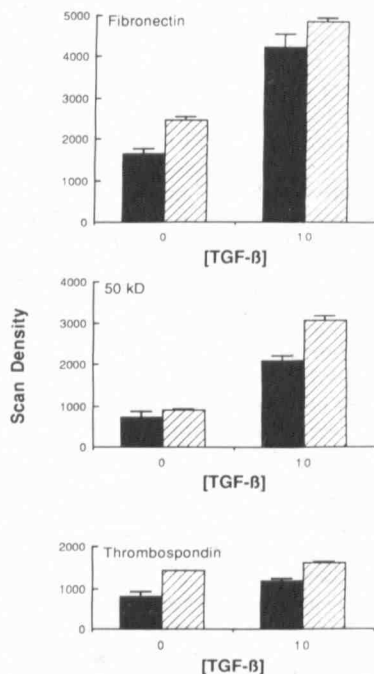


Figure 5. Graphical representation of the band densities, as determined by laser densitometry, of each of the three proteins of interest as a function of both [EGF] and [TGF-β]. A separate bar graph is given for each of the three proteins. The top graph is for fibronectin, the middle graph is for the 50-kD protein, and the bottom graph is for thrombospondin, as indicated. The solid bars represent [EGF] = 0, while striped bars represent [EGF] = 10 ng/ml. [TGF-β] is given on the abscissa of each graph. The ordinate is in arbitrary scan density units. The error bars indicate the standard errors of the means (n = 2). Where error bars are not visible, errors were too small to plot on this scale.

In order to detect any additional effect of EGF, these experiments were repeated using cells incubated in medium lacking EGF for 16 h. Human keratinocytes were then incubated for 22 h (10 of which were with label), as before. Each combination of [EGF] of 0 and 10, and [TGF-β] of 0 and 10 ng/ml was tested in duplicate. Autoradiographs from replicate experiments were identical. One of them is shown in Fig 4, and a graphical representation of the band densities is shown in Fig 5. Once again, TGF-β produced large increases in the secretion of metabolically labeled fibronectin and the 50-kD protein, while producing only a modest increase in thrombospondin secretion. This time, however, EGF also produced a small additional increase. Quantitation of these proteins using scintillation counting of the excised bands produced similar results (data not shown).

The results of analysis of variance are shown in Table I. The effects of TGF-β and EGF on each of the three proteins was highly significant. In the case of fibronectin and thrombospondin, the effects of TGF-β and EGF were purely additive; there was no interaction between the effects of the two factors. In the case of the 50-kD protein, however, there was statistically significant synergism between the effects of the two growth factors. This can be seen in Fig 5. At [TGF-β] = 0 there is no significant difference in the amount of the 50-kD protein between [EGF] = 0 and [EGF] = 10. At [TGF-β] = 10, however, there is a marked and statistically significant effect of [EGF]. Conversely, it can be stated that the effect of TGF-β is larger in the presence of EGF than in the absence of EGF.

The identity of the 50-kD protein was unknown. Because TGF-β stimulates collagen production in non-epithelial cell lines, it was of interest to determine whether this protein has collagenous domains. Samples of labeled medium from TGF-β stimulated cultures were subjected to either collagenase or pepsin digestion as detailed in "Materials and Methods." The 50-kD protein was completely digested by pepsin, but was not affected by collagenase (data not shown). Thus it is not a collagen. [Recent studies in our laboratory have identified the protein as plasminogen activator inhibitor of the endothelial cell type (unpublished data).]

The effects of TGF-β on cell and extracellular matrix expression of fibronectin were further studied by immunofluorescence. The results are shown in Fig 6. As is usual with this system of cultured keratinocytes, cells varied slightly in size, and somewhat more in degree of confluence within the same slide. Panels A and C show areas of relatively high confluence, while panels B and D show areas of lesser confluence, including some discrete cells. The pattern of extracellular staining depended on the degree of confluence. Around individual, discrete cells (as in the indicated cell near the center of panel D) or at the edges of groups of cells (such as the left-hand sides of panels A and B) fibronectin staining appeared as large, coarse, intertwining fibrils radiating away from the associated cells. Between more confluent cells (as in panel C) extracellular fibronectin appeared as thinner, shorter, and less organized fibrils. Cellular fibronectin was seen most prominently as granular perinuclear staining in all cells, whether discrete or confluent. Under all conditions there was cell-to-cell variation in the degree of cytoplasmic staining. TGF-β caused a dramatic increase both in cytoplasmic

Table I. P values (probabilities, as determined by two way analysis of variance, that differences greater than or equal to the ones observed could have occurred by chance) of the effects of [EGF] (0 vs. 10 ng/ml), [TGF-β] (0 vs. 10 ng/ml), and any interaction between the effects of [EGF] and [TGF-β] on the secretion of three proteins as tabulated. Quantitation was by densitometry. A value less than 0.05 is considered significant.

Secreted Proteins	Effects Of		
	[EGF]	[TGF-β]	Interaction
Fibronectin	0.019	<0.001	0.643
50 kD	0.009	<0.001	0.036
Thrombospondin	0.003	0.021	0.214

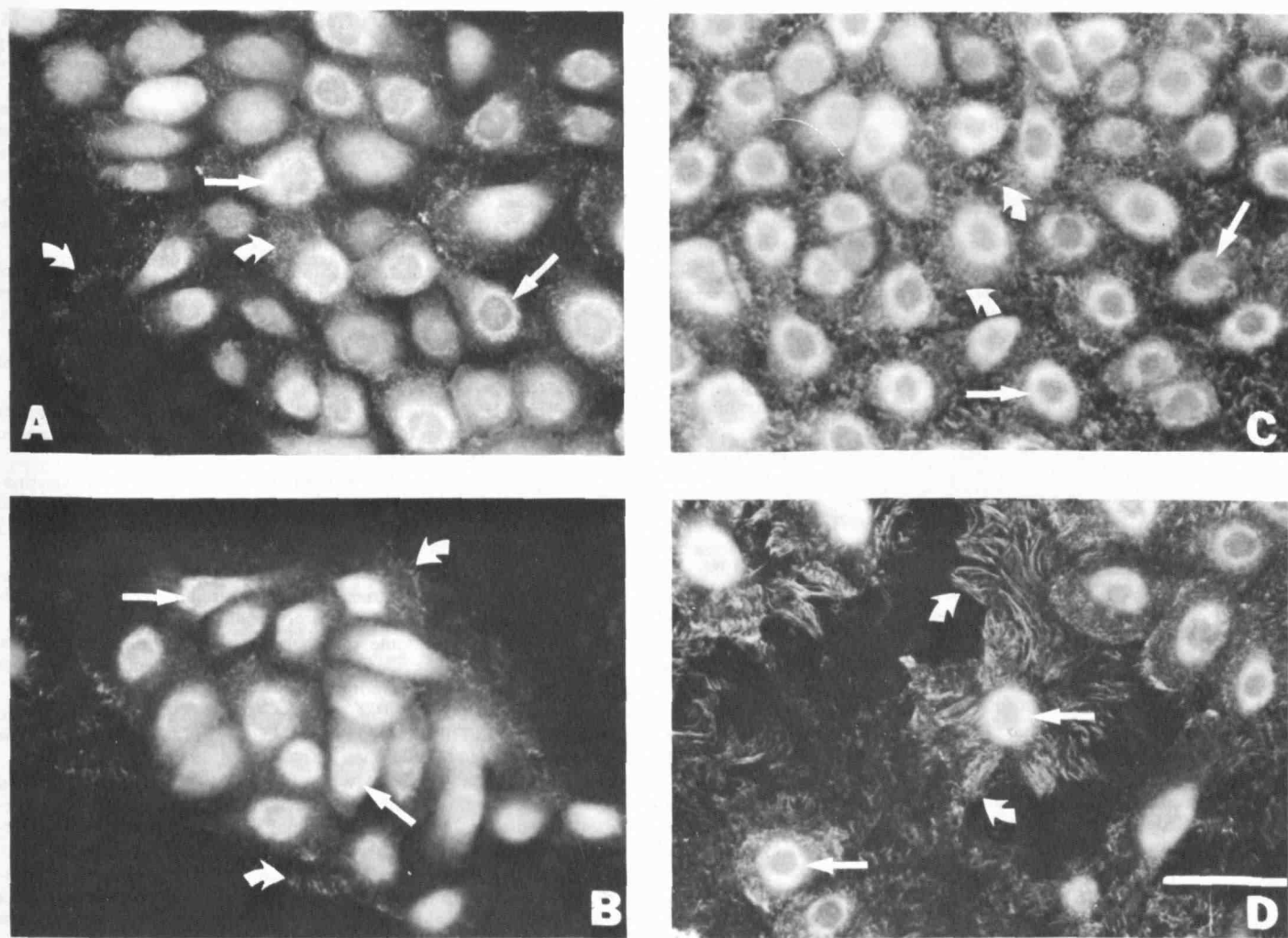


Figure 6. Immunofluorescent staining of fibronectin in unstimulated cells (A,B) and in TGF- β —stimulated cells (C,D). All photographs were exposed for 1 min under identical conditions of UV illumination and were taken using the same magnification. Straight arrows point to sites of cytoplasmic staining for fibronectin. Curved arrows point to sites of extracellular matrix staining for fibronectin. The bar, shown in panel D, represents a length of 50 microns on the original slide.

staining and in the extracellular matrix staining of fibronectin. For cytoplasmic staining, both the percentage of heavily staining cells, and the maximal intensity of staining was increased by TGF- β . The increase in extracellular staining caused by TGF- β was even more striking, especially around individual (nonconfluent) cells (as in panel D). Photometry of the slides showed luminescence values of 12 ± 2 ($n = 20$) for unstimulated cells and 18 ± 4 ($n = 19$) for cells stimulated with TGF- β (figures are mean \pm standard deviation, arbitrary units). This difference was significant with a p value of <0.001 .

DISCUSSION

We have demonstrated that TGF- β specifically induces the secretion of several proteins by human keratinocytes. The greatest increases detected were in fibronectin and in a 50-kD protein of unknown identity. The increased secretion of these metabolically labeled proteins is far in excess of the increase in secretion of all metabolically labeled proteins as a whole. Further, we have shown that cellular and extracellular matrix deposition of fibronectin is markedly increased by TGF- β stimulation. Thrombospondin synthesis is also increased, but this increase is actually less than the background increase in proteins other than fibronectin and the 50-kD protein.

The overall increase in secretion of metabolically labeled proteins

seen in response to TGF- β cannot be due to cellular proliferation. TGF- β has been shown, at these concentrations and under identical culture conditions [5], to arrest proliferation of human keratinocytes.

Our results are consistent with those obtained from mink lung and human lung carcinoma cell lines [7], and extend these findings to a non-transformed epithelial cell.

These results do not allow any conclusions concerning the functional significance of this effect. There are, however, some related observations which might allow some speculation. In normal skin, keratinocytes exhibit orderly differentiation and proliferation on a basement membrane consisting primarily of laminin, type IV collagen, and heparan sulfate proteoglycan. Except for slow upward movement from the basal layer, they do not migrate. In a healing wound, on the other hand, keratinocytes remain in a relatively undifferentiated state, they migrate actively, they do not proliferate as they migrate [22], and they migrate on a provisional matrix consisting largely of fibronectin [23]. Arrest of proliferation and differentiation, as well as stimulation of fibronectin secretion are all effects of TGF- β on human keratinocytes. Platelet alpha granules are a rich source of TGF- β [24]. Therefore TGF- β would be present in large quantities in an early wound. Thus one may speculate that TGF- β mediates several components of keratinocyte behavior in a healing wound.

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