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Original Article

TGFβ and TGFα, antagonistic effect *in vitro* on extracellular matrix accumulation by chick skin fibroblasts at two distinct embryonic stages

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ABSTRACT DECM macromolecules create a specific environment that participates in the control of cell proliferation and differentiation during embryogenesis. Quantitative and qualitative alterations in the ECM may depend on several growth factors that modify cell metabolism. Since transforming growth factor β (TGFβ) and α (TGFα) are abundantly expressed during embryonic development in organs in which epithelial-mesenchymal interactions occur, the aim of this study was to determine: a) the effect of TGFβ on the phenotype of 7 and 14 day chick embryo back skin (CEBS) fibroblasts by evaluating the neosynthesis of GAG, collagen and fibronectin; b) whether TGFα and TGFβ production, in particular TGFβ$_3$ and TGFβ$_4$, and the number of TGFβ receptors change during these two stages of embryonic development. The results show that the neosynthesis of ECM macromolecules, tested using radiolabelled precursors, is increased by TGFβ. The growth factor generally favours cellular accumulation more than secretion. As far as GAG is concerned, TGFβ has a greater stimulatory effect on sulphated GAG than on HA. Specific bioassay shows that TGFβ$_3$ and TGFβ$_4$ activity is higher in 7 day than 14 day CEBS fibroblasts. Moreover, TGFβ$_3$ and TGFβ$_4$ mRNA expression is increased in the first stages of development. Instead, the level of TGFα increases in successive developmental stages. Since TGFα stimulates the synthesis and secretion of HA, and HA binds and inactivates TGFβ, the greater quantity of HA in 14 day fibroblasts may contribute to reducing the TGFβ effect. Overall our data suggest that the production of TGFβ and TGFα are age-dependent and that the balance between the two growth factors may be a mechanism for controlling skin differentiation.

KEY WORDS: *TGFβ$_3$, TGFβ$_4$, TGFα, chick embryo skin fibroblasts, extracellular matrix*

Introduction

During embryonic development intensively interactive processes take place; i.e., epithelial-mesenchymal interactions (Chuong, 1993). In this connection, avian skin has been largely used since it presents two interesting aspects: epidermis differentiation and plumar bud formation.

In vivo 7 day back skin presents a bistratified epithelium that overlies a thin dermis. As an initial step in the formation of the feathers, mesenchymal condensation appears at intervals and provides two dorsal rows of feather buds on either side of the spinal cord. At 14 days numerous feather filaments had differentiated, curved in a caudal direction and covered by multilayered epithelium, whereas epithelium of interplumar skin is three layered. Two distinct populations of fibroblasts are present: those which form the dermal papilla and the interplumar ones. At 17 days of incubation the interplumar epithelium becomes multilayered and undergoes keratinization (Hamilton, 1965).

The expression of specific ECM macromolecules is correlated to developmental age: relative accumulation of sulphate GAG in the early stages of development and relative accumulation of HA in the advanced ones (Pane *et al.*, 1974; Kawamoto and Nagay, 1976). Therefore, specific accumulation and modifications in the

Abbreviations used in this paper: CEBS, chick embryo back skin; ECM, extracellular matrix; TGFβ, transforming growth factor beta; TGFα, transforming growth factor alpha; GAG, glycosaminoglycans; HA, hyaluronic acid; HS, heparan sulphate; CS, chondroitin 4-6 sulphate; DS, dermatan sulphate; CM, conditioned medium.

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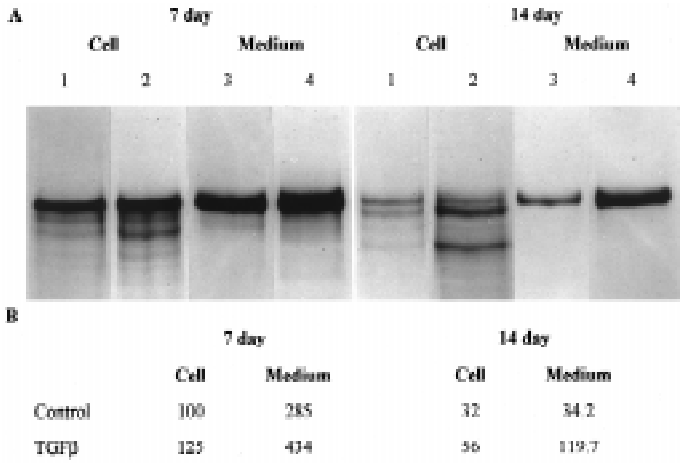

Fig. 1. Seven and 14 day CEBS fibroblasts were maintained in 199 with or without TGFβ (4 ng/ml) for 24 h, ^{35}S-methionine (20 μCi/ml) was added during the last 3 h. *Then cells and media were subjected to gelatin-Sepharose affinity chromatography. The isolated fibronectin was analysed by electrophoresis on 6% SDS-polyacrylamide slab gels and fluorography.* **(A)** *Autoradiogram film of the gels. The samples are as follows: lanes1 and 3, no additions; lanes 2 and 4, 4 ng/ml TGFβ.* **(B)** *Quantitation of the fibronectin synthesised and secreted by 7 and 14 day CEBS fibroblasts with or without TGFβ. The fluorographs were analysed by scanning densitometry. The absolute counts were converted to percent of control values assuming 100% as the level of untreated 7 day CEBS fibroblasts.*

composition of ECM macromolecules (such as GAG, collagen and fibronectin) under the control of the growth factors are considered regulatory in epithelial histogenesis and growth factor activity (Carinci e*t al.,* 1978, 1986, 1991; Becchetti *et al.,* 1984, 1988; Knudson and Toole, 1985; Burgess and Maciag, 1989; Evangelisti *et al.,* 1989; Locci *et al.*, 1993, 1995; Bodo *et al.,* 1994).

TGFβ, in particular, is an ubiquitous factor which is abundantly expressed during embryonic development in organs where interactions between the epithelium and mesenchyme take place (Pelton *et al.,* 1989; Schmid *et al.,* 1991; Sanders *et al.,* 1993). This factor regulates the accumulation of the macromolecules that make up the ECM through a dual mechanism: it stimulates synthesis of ECM components and inhibits those enzymes that favour its degradation (Rizzino, 1988; Roberts *et al.,* 1988; D'Angelo and Greene, 1991; Locci *et al.,* 1993).

There are at least five highly homologous, yet distinct isoforms of TGFβ: TGFβ_1, β_2 and β_3 from several species that include mammals (Roberts and Sporn, 1990), TGFβ_4 from chickens where TGFβ_1 is absent (Jakowlew *et al.,* 1988, 1994) and TGFβ_5 from amphibians (Kondaiah *et al.,* 1990). TGFβ binds to receptors on the cell surface, designated type I, II and III, of about 60, 90 and 280 kDa respectively. The type III receptor is a membrane proteoglycan, called betaglycan, that binds the growth factor but does not transduce the signal. Betaglycan facilitates the binding of the growth factor to type I and II receptors. TGFβ binds to type II receptors and the type II TGFβ receptor complex is recognised by type I receptors, which transduce the signal through a cytoplasmic serine/threonine kinase domain. (Wrana *et al.,* 1992; Ebner *et al.,* 1993a,b; Lopez-Casillas *et al.,* 1993).

Transforming growth factor α (TGF α) is a single polypeptide sharing structural and functional homology with epidermal growth factor (Derynck, 1988; Massagué, 1990). TGFα is derived from membrane-anchored precursor. The growth factor is biologically active via its ability to interact with the epidermal growth factor receptor and exerts a variety of similar biological activities both *in vivo* and *in vitro*. It is known to affect epithelial and mesenchymal cell proliferation, migration and differentiation (Harvey *et al.,* 1991; Mogi *et al.,* 1995).

We previously demonstrated that chick embryo back skin (CEBS) fibroblasts produce both TGFα and TGFβ, which influence GAG accumulation, and hypothesised that these two growth factors are differentially modulated during skin development (Locci *et al.,* 1993). In the present research, we examined the ability of CEBS fibroblasts at 7 and 14 days of foetal development to synthesise ECM macromolecules, such as GAG, collagen and fibronectin in response to TGFβ addition, in radiolabelled precursor incorporation experiments. We also analysed TGFβ activity, TGFβ receptors and TGFβ mRNA expression during the same embryonic periods. Lastly, since skin fibroblasts produce both TGFα and TGFβ, which exert different effects on ECM macromolecule accumulation, we assayed TGFα activity at 7 and 14 days of foetal development to test the hypothesis that a programmed modulation of the two growth factors may promote a specific ECM composition that controls skin differentiation.

Results

GAG biosynthesis

Seven day CEBS fibroblasts accumulated about the same quantity of GAG in cells and media, whereas 14 day fibroblasts accumulated more GAG in the media and so secreted a higher percentage of GAG. Moreover, total GAG synthesis (cells plus media) was greater in 7 than 14 day fibroblasts (Locci *et al.,* 1993). When TGFβ_1 was added, GAG increased in cells and media at both times (Table 1). Moreover, the increase was greater at 14 days (about 4.4-fold in cells and 3.2-fold in media) than at 7 days (2 fold in cells, 1.9 fold in media). Four μg/ml TGFβ_1 was chosen because the growth factor shows a dose increasing effect up to this concentration; furthermore different TGFβ isoforms have about the same effects on ECM macromolecule accumulation (unpublished data).

TABLE 1

EFFECTS OF TGFβ ON 3H-GLUCOSAMINE INCORPORATION INTO 7 AND 14 DAY CEBS FIBROBLASTS

	Cellular GAG	Extracellular GAG	Total GAG	%secreted GAG
7 day				
Control	18,432 ± 210	19,508 ± 990	37,940	51
TGFβ	38,304 ± 1,877*	36,890 ± 1,964*	75,194	49
14 day				
Control	8,914 ± 388	21,210 ± 443	30,124	70
TGFβ	38,980 ± 1,830*	69,100 ± 3,767*	108,080	64

Values are expressed as c.p.m./culture and represent the mean ± SD of four replicates from a typical experiment. Similar results were obtained in three independent experiments. Significance versus control *P < 0.001.

Chromatography sequentiation of GAG secreted into the media revealed four classes: HA, HS, CS and DS. The GAG class most expressed in control cells was HA (Table 2). TGFβ_1 induced a rise in all four GAG classes, but particularly in CS, at both 7 and 14 days, and DS at 7 days. Consequently, the percentage of HA and HS was lower than in controls.

Collagen biosynthesis

There were no significant differences in the total (cells plus media) collagen accumulation of 7 and 14 day fibroblasts cultured for 24 h without serum (Table 3). Both 7 and 14 day fibroblasts accumulated less collagen in cells than media. Addition of TGFβ_1 caused a marked increase in collagen, which was greater at 14 days (3.1 times in cells, 2.7 in media vs. controls) than at 7 days (2.2 times in cells, 2 fold in media vs. controls).

Fibronectin biosynthesis

Densitometric analysis of fluorographs of fibronectin showed that the amount of the protein synthesised and secreted into the culture media was much higher in 7 day than 14 day fibroblasts (Fig. 1A,B). Moreover, whereas almost identical quantities of fibronectin were found in the cells and media of 14 day fibroblasts, in 7 day fibroblasts the quantity of fibronectin accumulated in the media was almost 3-fold that present in the cells. TGFβ_1 increased glycoprotein more in media than in cells in both embryonal populations. The growth factor stimulated increase was more at 14 than at 7 days.

TGFβ_3 and TGFβ_4 activity

Antibody specificity is shown in Figure 2A. Compared to control (Fig. 2B, lane 1), CM (20 μg) of 7 day CEBS fibroblasts inhibited the proliferative response of CCL-64 by 73% (Fig. 2B, lane 2). The fact that anti-TGFβ_1 administration had no effect on the proliferative response of CCL-64 is confirmation that chickens did not express TGFβ_1 (Fig. 2B, lane 3). Addition of anti-TGFβ_2 to the CM of 7 day CEBS fibroblasts neutralised 62% of TGFβ activity, demonstrating that TGFβ_2 activity was 11% and TGFβ_3 plus TGFβ_4 activity 62% (Fig. 2B, lane 4). When anti-TGFβ_2 and anti-TGFβ_3 were added, they reduced TGFβ activity by 55%, showing that TGFβ_4 activity was 55% (Fig. 2B, lane 5), thereby indicating that 7% was TGFβ_3 activity. In fact, the TGFβ_3 anti-body neutralised approximately 7% of TGFβ activity (Fig. 2B, lane 6).

TABLE 2

EFFECTS OF TGFβ ON 3H-GLUCOSAMINE INCORPORATION INTO GAG CLASSES IN THE MEDIUM BY 7 AND 14 DAY CEBS FIBROBLASTS

	HA	HS	CS	DS	Total GAG
7 day					
Control	7,705 (39.5)*	3,463 (17.5)	5,404 (28)	2,936 (15)	19,508
TGFb	9,908 (27)	4,479 (12)	14,598 (39.5)	7,905 (21.5)	36,890
14 day					
Control	13,341 (63)	1,892 (9)	4,065 (19)	1,912 (9)	21,210
TGFb	32,074 (46.5)	4,407 (6.5)	26,379 (38)	6,240 (9)	69,100

Values are expressed as c.p.m./culture. *Percentage of secreted GAG.

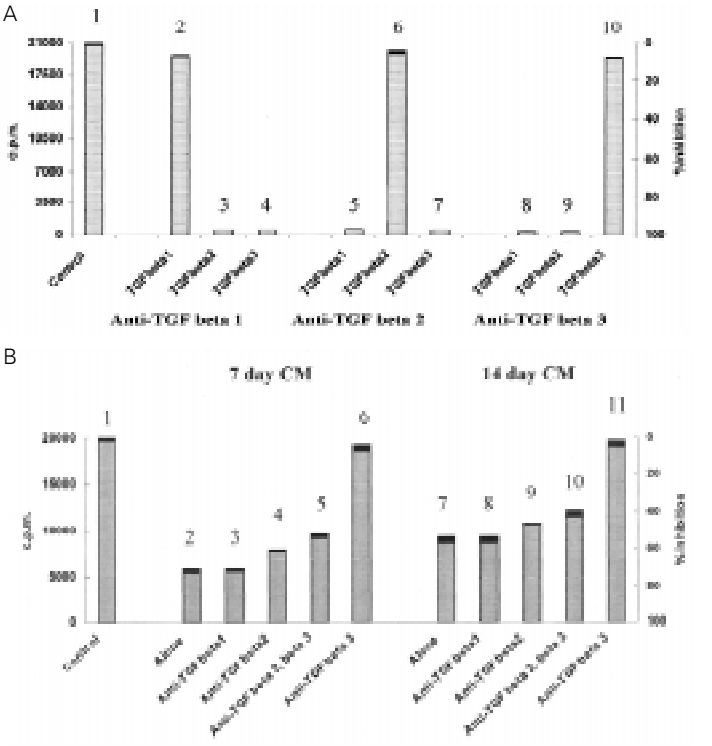

Fig. 2. Immunoneutralisation analysis using TGFβ_1, β_2, β_3 and anti-TGFβ_1,β_2,β_3 specific antibodies in the CCL-64 bioassay (A) . *Control (line 1); TGFβ_1, TGFβ_2, TGFβ_3 in presence of anti-TGFβ_1 (lanes 2,3,4); TGFβ_1, TGFβ_2, TGFβ_3 in presence of anti-TGFβ_2 (lane 5,6,7); TGFβ_1, TGFβ_2, TGFβ_3 in presence of anti-TGFβ_3 (lane 8,9,10). Data shown are from a representative experiment. Means and standard deviations were obtained from triplicate cultures.*

(B) *Immunoneutralisation analysis of TGFβ activity in CM of 7 and 14 day CEBS fibroblasts using TGFβ_1, β_2, β_3 and TGFβ_1, β_2, β_3 specific antibodies and the CCL-64 bioassay. Control (line 1); 7 or 14 day CM (lanes 2,7); 7 or 14 day CM + anti-TGFβ_1 (lanes 3,8); 7 or 14 day CM + anti-TGFβ_2 (lane 4,9); 7 or 14 day CM + anti-TGFβ_2 + anti-TGFβ_3 (lanes 5,10); 7 or 14 day CM + anti-TGFβ_3 (lane 6, 11). Data shown are from a representative experiment. Means and standard deviations were obtained from triplicate cultures.*

CM (20 μg) of 14 day CEBS fibroblast inhibited the proliferative response of CCL-64 by 56% (Fig. 2B, lane 7), showing that CM of 7 day CEBS fibroblasts had a higher inhibitory activity than 14 day CEBS fibroblasts. Addition of anti-TGFβ_1 to CCL-64 cells (Fig. 2B, lane 8) showed that TGFβ_1 was not expressed. Anti-TGFβ_2 neutralised 47% of TGFβ activity in the CM of 14 day CEBS fibroblasts, TGFβ_2 activity was therefore 9% and TGFβ_3 plus TGFβ_4 activity 47% (Fig. 2B, lane 9). As TGFβ activity was neutralised to 43% by addition of anti-TGFβ_2 and anti-TGFβ_3, TGFβ_4 activity was 43% (Fig. 2B, lane 10). Addition of TGFβ_3 anti-body neutralised 4% of TGFβ activity, therefore TGFβ_3 activity was 4% (Fig. 2B, lane 11). The TGFβ concentration, assayed by ELISA kit, was 1.145 ng/culture in CM of 7 day CEBS fibroblasts and 0.755 ng/culture in CM of 14 day CEBS fibroblasts. The amount of single isoforms was 0.172, 0.109 and 0.864 ng/culture for TGFβ_2, β_3 and β_4 respectively in 7 day CEBS fibroblasts, 0.121, 0.054 and 0.580 ng/culture for TGFβ_2, β_3 and β_4 respectively in 14 day CEBS fibroblasts.

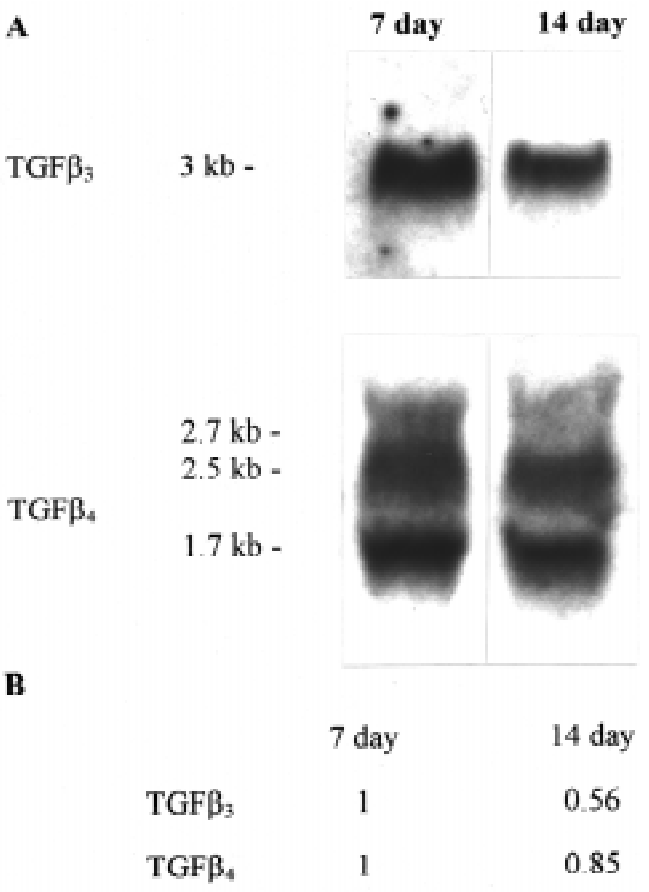

Fig. 3. *TGFβ_3 and TGFβ_4 mRNA levels by 7 and 14 day CEBS fibroblasts.* **(A) Nothen blot analysis.** *Relatively equal amounts of total RNA (20 μg) were loaded in each lane and hybridised with chicken TGFβ_3 and TGFβ_4 cDNA probes. Size of TGFβ_3 mRNA is shown as 3 kb. The positions of TGFβ_4 mRNA transcript are shown as 2.7, 2.5 and 1.7 kb. For normalisation purposes, the same nylon membranes were stripped and rehybridised successively with a ^{32}P-labelled GAPDH cDNA probe (data not shown). Similar results were obtained in three separate experiments.* **(B)** *The mRNA levels were determined on the Northern blots (A) and quantified by laser scanning densitometry. The values indicate mRNA abundance expressed as relative densitometric units/20 μg total RNA analysed/lane and corrected for the abundance of GAPDH mRNA in the same samples. Similar results were obtained in three separate experiments.*

TGFα activity assay

CEBS fibroblasts spontaneously produce TGFα. The ELISA test demonstrated that the amount of TGFα secreted into the CM (Table 4) was considerably higher in 14 than in 7 day CEBS fibroblasts. The concentration of TGFα secreted was 0.116 ng/culture in 7 and 0.205 ng/culture in 14 day CEBS fibroblasts.

TGFβ_3 and β_4 mRNA expression

Northern blotting was performed to assess the relationship between the TGFβ protein level and TGFβ mRNA. TGFβ_3 and TGFβ_4 cDNAs were used because they are abundantly expressed in chick embryo. In high stringency conditions, the TGFβ_3 probe hybridised with mRNA by about 3 kb (Fig. 3A), the TGFβ_4 probe hybridised with mRNA by about 2.7, 2.5 and 1.7 kb (Fig. 3A) isolated from both of the cell culture populations studied. Densitometric analysis showed that TGFβ_3 and TGFβ_4 mRNA were more expressed in 7 than in 14 day fibroblasts (Fig. 3B). The values were normalised to GAPDH mRNA levels.

TGFβ receptors

The binding of TGFβ to 7 and 14 day CEBS fibroblasts was assayed and the binding data subjected to Scatchard analysis. A typical set of binding curves and the corresponding Scatchard analysis are shown for 7 day fibroblasts in Figure 4 and for 14 day fibroblasts in Figure 5. Since TGFβ isoforms bind the same receptors and only iodinated TGFβ_1 is commercially available, we used this isoform. Binding isotherms with various concentrations of ^{125}I-TGFβ_1 and native TGFβ_1 indicated that saturation of TGFβ receptors at 4°C was achieved at 0.20-0.25 nM TGFβ in 7 day and at 0.25-0.30 nM TGFβ in 14 day CEBS fibroblasts. Scatchard analysis gave a linear plot characteristic of a single class of high affinity binding sites with about 13,798 receptors/cell and a dissociation constant (Kd) of 151 pM in 7 day CEBS fibroblasts (Fig. 4), and about 13,196 receptors/cell with a Kd of 109 pM in 14 day CEBS fibroblasts (Fig. 5).

Unlabelled TGFβ_1 (4 ng/ml) was added to parallel sets of 7 and 14 day fibroblast cultures to allow internalisation of the ligand-receptor complex. The binding capacity was compared with that of the cells not previously exposed to TGFβ_1. As the Scatchard graph shows, the receptor/cell ratio was 11,082 with a Kd of 153 in 7 day fibroblasts (Fig. 6) and 11,142 with a Kd of 111 in 14 day fibroblasts (Fig. 7). Therefore, pre-exposure to TGFβ reduced the number of receptors on 7 day fibroblasts by about 20% and on 14 day fibroblasts by about 16% (Figs. 6 and 7). The TGFβ receptors show (Fig. 8) two bands of about 300 and 60 kDa, which correspond to type I and I receptors respectively. Type II receptors were not detected. Densitometric analysis revealed no differences in receptor type expression of 7 and 14 day fibroblasts. In the presence of an excess of unlabelled TGFβ_1, the bands disappeared, thereby confirming that the receptors bound the growth factor (data not shown).

Discussion

The present study forms a part of a long ongoing research designed to analyse the phenotype of 7 and 14 day-old CEBS

TABLE 3

EFFECTS OF TGFβ ON 3H-PROLINE INCORPORATION IN CELLULAR AND EXTRACELLULAR COLLAGEN IN 7 AND 14 DAY-OLD CEBS FIBROBLASTS

	Cellular collagen	Extracellular collagen	Total collagen
7 day			
Control	2,700 ± 124	4,550 ± 356	7,250
TGFβ	*5,930 ± 103	*9,300 ± 474	15,230
14 day			
Control	2,051 ± 358	5,391 ± 460	7,442
TGFβ	*6,304 ± 237	*14,613 ± 691	20,917

Values are expressed as c.p.m./culture and represent the mean ± SD of four replicates from a typical experiment. Similar results were obtained in three independent experiments.Significance versus control *P < 0.001.

fibroblasts and the capacity of TGFα and β to modulate the composition of the ECM in these two developmental stages *in vitro*.

Our results show that the pattern and amount of collagen are similar in 7 day and 14 day CEBS fibroblasts, whereas GAG and fibronectin levels are higher at 7 than 14 days. Cellular and extracellular macromolecule accumulation was differently modulated by TGFβ treatment. The growth factor induced increases in collagen, fibronectin and GAG; the increase was greatest for GAG, particularly sulphated GAG, and higher at 14 than 7 days.

To verify whether or not the growth factor is differently expressed in 7 and 14 day CEBS fibroblasts, it was tested by determining $TGF\beta_3$ and β_4 isoform activity and mRNA expression. Both $TGF\beta_3$ and β_4 activity, and $TGF\beta_3$ and $TGF\beta_4$ mRNA expression, were higher at 7 than 14 days thus indicating that in the earlier stages of development the increase of $TGF\beta_3$ and β_4 expression translated with the release of the factor in the medium. Since TGFβ induces an increase in ECM macromolecule accumulation (Sporn and Roberts, 1992), the reduction in TGFβ mRNA transcripts and TGFβ activity in 14 day CEBS fibroblasts might explain the decrease in collagen, fibronectin and sulphated GAG observed in this developmental stage compared to 7 days.

The differential redistribution of ECM components could contribute to the new signals required for morphogenesis. Cell interaction with fibronectin affects cytoskeletal organisation, cell mobility and matrix remodelling (Silver *et al.,* 1981; Damsky and Werb, 1992). When associated with type I collagen fibrils, the glycoprotein plays an important role in morphogenic events that occur during organogenesis and cytodifferentiation, which suggests a role in cell phenotype modulation (McDonald, 1988; Arend, 1991; Moursi *et al.,* 1996). The higher fibronectin expression in 7 day-old fibroblasts, compared to 14 day-old ones, may be related to the successive feather bud development (Norton and Hynes, 1987; Ffrench-Constant and Hynes, 1988, 1989). Moreover, fibronectin may modulate the organisation of ECM components such as GAG and collagen that allow signalling by TGFβ.

The studies, designed to ascertain whether the number and type of TGFβ membrane receptors differ at the two embryonic stages investigated, showed that both cell populations proved to have about the same number of receptors per cell and a very similar Kd, which agrees with the findings of others (Wakefield *et al.,* 1987) who reported that the fibroblast response to TGFβ is not modulated by the number of receptors per cell.

Cross-linking studies with iodinate $TGF\beta_1$ revealed type I receptors and betaglycan, but failed to detect type II receptors, even if both type I and II receptors are required to mediate the biological effects of TGFβ ligands (Wrana *et al.,* 1992; Franzen *et al.,* 1993)

TABLE 4

TGFα CONCENTRATION IN CM OBTAINED FROM 7 AND 14 DAY CEBS FIBROBLASTS QUANTIFIED BY RIA KIT

	TGFα pg/culture
7 day CM	0.116 ± 0.003
14 day CM	0.205 ± 0.011*

Mean ± SD of four replicates from a typical experiment. Similar results were obtained in three independent experiments. Significance *P < 0.001.

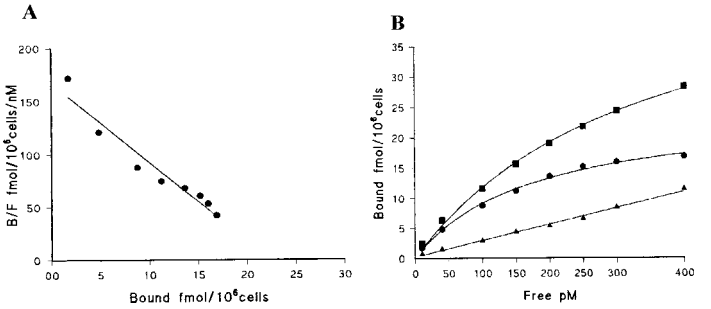

Fig. 4. Scatchard plot of TGFβ binding to 7 day CEBS fibroblasts. (A) *Specifically bound (B) and free (F) ligand were measured as described in Materials and Methods.* **(B)** *Binding of ^{125}I-TGFβ to specific receptors of 7 day CEBS fibroblasts. The TGFβ specifically bound (Σ) is the difference between the total (■) and non specific (▲) binding. Each point is the mean of three determinations that generally differed by <5%.*

and both must be present in CEBS fibroblasts. Other investigators failed to detect type II receptors but documented type II receptor mRNA expression (Franzen *et al.,* 1993; Takeuchi *et al.,* 1995).

The studies carried out on fibroblasts precultured in the presence of unlabelled growth factor to answer the question of whether high concentrations of the growth factor exert an influence on the number of receptors showed that pre-incubation with unlabelled TGFβ rapidly reduced subsequent binding of the radio-ligand. Others have reported similar findings (Centrella *et al.,* 1996; Wells *et al.,* 1997) and suggested that rapid turnover of TGFβ receptors, particularly type II, might account for this finding (Koli and Arteaga, 1997; Wells *et al.,* 1997).

It has been speculated that variations in cellular effects might result from different intracellular response systems, as well as from changes in the proportion of type I and II TGFβ receptors (Chen *et al.*, 1993; Takeuchi *et al.*, 1995). The rapid turnover of TGFβ receptors potentially allows receptor numbers to change quickly in response to various stimuli, an important characteristic for a growth factor with wide ranging effects on differentiation.

During epidermal differentiation the relative accumulation of HA in the ECM, rather than sulphated GAG, is considered regulatory in skin differentiation (Carinci *et al.,* 1978; Becchetti *et al.,* 1984; Locci *et al.,* 1992; Bodo *et al.*, 1993; Sorrell *et al.,* 1996). In fact, the extracellular GAG pattern of the mesenchyme both *in ovo* and *in*

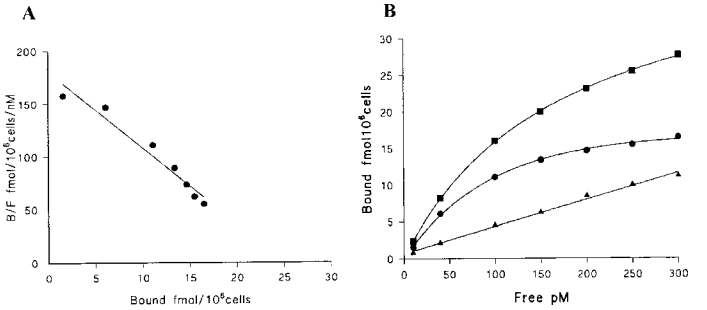

Fig. 5. Scatchard plot of TGFβ binding to 14 day CEBS fibroblasts. (A) *Specifically bound (B) and free (F) ligand were measured as described in Materials and Methods.* **(B)** *Binding of ^{125}I-TGFβ to specific receptors of 14 day CEBS fibroblasts. The TGFβ specifically bound (Σ) is the difference between the total (■) and non specific (▲) binding. Each point is the mean of three determinations that generally differed by <5%.*

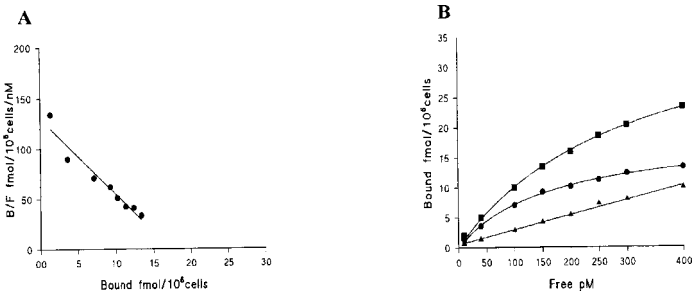

Fig. 6. Scatchard plot of TGFβ binding to 7 day CEBS fibroblasts treated with 4 ng/ml of TGFβ (A). *Specifically bound (B) and free (F) ligand were measured as described in Materials and Methods.* **(B)** *Binding of ^{125}I-TGFβ to specific receptors of 7 day CEBS fibroblasts treated with 4 ng/ml of TGFβ. The TGFβ specifically bound (Σ) is the difference between the total (■) and non specific (▲) binding. Each point is the mean of three determinations that generally differed by <5%.*

vitro is related to the nature of the evolution of the epithelium: prevalence of HA during the keratinization process, prevalence of CS during the feather bud formation. In agreement with these observations, 7 day-old CEBS fibroblasts accumulated more sulphated GAG, particularly CS, compared to 14 day fibroblasts, while 14 day fibroblasts accumulated more HA. During dermal condensation and feather germ development there is a high expression of TGFβ, which favours CS accumulation (Ting-Berreth and Chuong, 1996).

We previously demonstrated that TGFα is produced by CEBS fibroblasts and that, when added to the cells, its effect on single GAG class accumulation is the opposite to that of TGFβ (Locci *et al.,* 1993). TGFα enhances HA more than sulphated GAG production. To support the hypothesis that the sequence of events that leads to the differentiation of the epidermis are connected with a drop in TGFβ activity and a parallel rise in TGFα activity, we measured TGFα activity in both 7 and 14 day CEBS fibroblasts. We found that TGFα activity increased between foetal day 7 and 14. Other investigators have also reported low TGFα and high TGFβ in the first stages of development, but a reversed situation in later stages (Baskin *et al.,* 1996). The increased TGFα in the 14 day fibroblasts raises the level of HA in the ECM (Locci *et al.,* 1993). Because TGFβ binds to HA, which inactivates the growth factor and protects it from rapid enzymatic degradation, the higher levels of HA in the 14 day fibroblasts' ECM suggests that a portion of the TGFβ in the ECM may be inactivated by HA (Locci *et al.,* 1992; 1995).

The obtained data suggest a model in which TGFα and TGFβ isoforms are differentially modulated during development and that the balance between the amount and secretion of TGFα and TGFβ may represent a homeostatic mechanism aimed at controlling normal skin differentiation. Preliminary studies on 7 day skin explants cultured *in vitro* for further 5 days in serum-free medium show that TGFα addition favours epithelium keratinization, whereas TGFβ addition favours feather bud elongation (unpublished data). Further research into organotypic cultures are in progress to verify whether the balance between these two growth factors, specific ECM accumulation and skin differentiation are closely correlated.

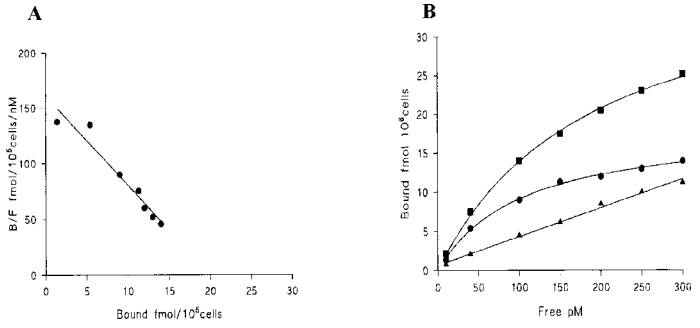

Fig. 7. Scatchard plot of TGFβ binding to 14 day CEBS fibroblasts treated with 4 ng/ml of TGFβ (A). *Specifically bound (B) and free (F) ligand were measured as described in Materials and Methods.* **(B)** *Binding of ^{125}I-TGFβ to specific receptors of 14 day CEBS fibroblasts treated with 4 ng/ml of TGF-β. The TGFβ specifically bound (Σ) is the difference between the total (■) and non specific (▲) binding. Each point is the mean of three determinations that generally differed by <5%.*

Materials and Methods

Cell cultures

Back skin fragments were removed from 7 and 14 day chick embryo staged according to the Hamburger-Hamilton Table (Hamburger and Hamilton, 1951), cut into small pieces and dissociated in 0.25% trypsin (1/250; DIFCO Laboratories, Detroit, MI, USA) in Ca^{++} and Mg^{++} free Hanks' balanced salt solution at room temperature for 30 min. The dissociated cells were filtered through a nylon mesh, centrifuged (350g, 10 min), then washed with phosphate buffered saline (PBS) pH 7.4 and suspended in medium 199 (GIBCO, Brl, Grand Island, NY) plus 10% foetal calf serum (GIBCO). Cell suspension ($1x10^6$ cells/ml) was plated in Nunclon wells (Denmark) and incubated in a humidity saturated atmosphere (5% CO_2, 37°C) to obtain confluent monolayer cultures. The absence of a suitable medium prevents the adhesion of epithelial cells (Emerman and Wilkinson, 1990). After 48 h a monolayer of confluent fibroblasts was obtained. There were about 2.106 cells/culture both in treated and untreated cultures.

Preparation of conditioned medium (CM)

Confluent 7 and 14 day (CEBS) fibroblasts were washed with physiological solution and cultured for 12 h in serum free medium 199. This medium was discarded to avoid contamination by seric factors. Cells were cultured for the next 24 h in 199 alone. The conditioned medium (CM), collected from 7 and 14 day CEBS fibroblasts was centrifuged for 10 min at 350g to remove cell debris, dialysed, lyophilised and dissolved in the medium used for TGFα and TGFβ activity assay.

TGFβ_3 and TGFβ_4 activity assay

The level of TGFβ was quantified in the CM of 7 and 14 day CEBS fibroblasts using an ELISA kit according to the manufacturer's instructions.

In a second set of experiments, TGFβ activity was assessed as the ability to inhibit Mv-1-Lu mink lung epithelial cells (ATCC/CCL-64) proliferative response using ^{3}H-thymidine incorporation bioassay as described by Kim and Schomberg (1989) and Mulheron *et al.* (1992). Standard curve was run to determine TGFβ concentration. Briefly, CM was dissolved in MEM containing 10% FCS and 1% non essential amino acids. $TGF\beta_3$ and $TGF\beta_4$ activity in CM were determined by measuring the inhibition of ^{3}H-thymidine incorporation (s.a. 6.7 Ci/mmole, NEN Du Pont de Nemours, RFG) by CCL-64 cell line. CCL-64 cells, seeded in 96-well microtiter plates at low cell density (1.10^4 cell/well), were subjected to either no addition (control), or added with 50 μl of CM (protein concentration 20 μg) in presence of neutralising anti-$TGF\beta_1$, or anti-$TGF\beta_2$, or anti-$TGF\beta_3$, or anti-$TGF\beta_2$ plus anti-$TGF\beta_3$ anti-bodies. All cultures were maintained for 72 h and pulsed with 2.5 μCi/ml of ^{3}H-thymidine for the last 6 h of incubation. Cells were harvested with a semiautomated cell harvester and the amount of ^{3}H-thymidine incorporated was determined using the standard liquid scintillation method. A complete characterisation of the anti-bodies used in both assays was done as described by Mulheron *et al.* (1992). Purified

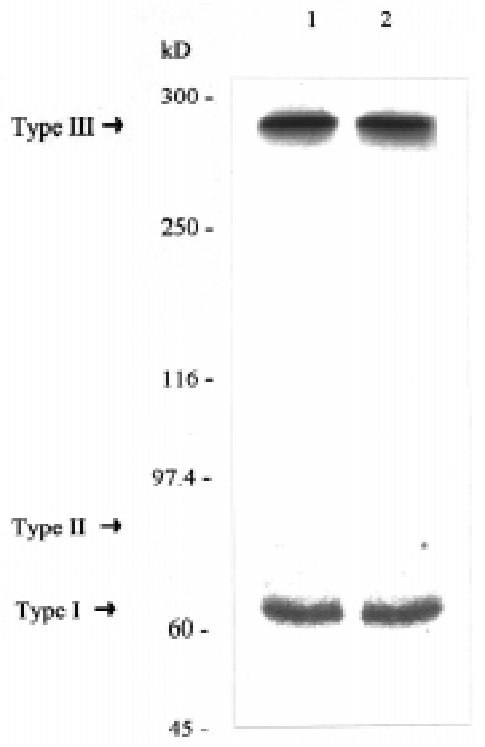

Fig. 8. Affinity labelling of 7 (lane 1) and 14 day (lane 2) CEBS fibroblasts with ^{125}I-TGFβ. *Cell proteins were separated by SDS-PAGE under reducing conditions and the labelled proteins were detected by autoradiography. Labelled bands corresponding to receptors I, II and betaglycan are indicated.*

TGFβ_1, β_2, β_3 were purchased from GIBCO and specific neutralising antibodies from RD Systems (Minneapolis, MN, USA). Anti-TGFβ_1 and anti-TGFβ_3 were produced from a murine hybridoma, anti-TGFβ_2 from goat immunised with purified, recombinant human TGFβ_1, β_3 and β_2 respectively.

TGFα activity assay.

The level of TGFα was quantified in the CM of 7 and 14 day CEBS fibroblasts with a specific radioimmunoassay ELISA kit (Nuclear Laser Medicine, Milano, Italy) according to the manufacturer's instructions. Standard curve was run to determine TGFα concentration in the unknown samples. All samples were assayed in triplicate. Results were expressed as pg/culture. Purified TGFα was purchased from Sigma-Aldrich (Milano, Italy).

Newly synthesised GAG isolation and identification

Confluent 7 and 14 day CEBS fibroblasts were maintained for 24 h in 199 or 199 plus TGFβ_1 (4 ng/ml) containing 5 μCi/ml of ^{3}H-glucosamine hydrochloride (s.a. 36.7 Ci/mmol, NEN). At the end of the incubation time, cells and media were recovered separately. Cells were scraped in 1 ml ice-cold 0.1 M Tris-HCl and 1.5 mM $CaCl_2$ (pH 8.2) and lysed by sonicating with six 10 sec bursts using a B15P model Branson sonicator. Media were dialysed, lyophilised and dissolved in the above buffer. Lysates and media were treated as previously described (Locci *et al*., 1992).

Analysis of collagen

Confluent 7 and 14 day CEBS fibroblasts were cultured for 24 h in MEM without serum supplemented with L-ascorbic acid (50 μg/ml), β-aminopropionitrile fumarate (50 μg/ml) and 10 μCi/ml of ^{3}H-proline (s.a. 35 Ci/mmol, NEN) with or without TGFβ_1. Collagen was extracted according to Webster and Harvey (1979). The specificity of the assay was determined by susceptibility of the precipitated collagen to purified bacterial collagenase.

Analysis of fibronectin

Confluent 7 and 14 day CEBS fibroblasts were cultured for 24 h in MEM with or without TGFβ_1 and labelled with 20 μCi/ml of ^{35}S-methionine (s.a.>1,000 Ci/mmol, Amersham International, England, U.K.) during the last 3 h of incubation. After biosynthetic labelling, fibronectin was isolated from media as described by Engvall and Ruoslahti (1977). Samples were subjected to SDS-PAGE analysis, followed by densitometric quantitation of fluorographs. The absolute counts were converted to percentage of control value assuming 100% as the level of untreated cell pool of 7 day CEBS fibroblasts.

TGFβ receptor assay

Sub-confluent 7 and 14 day CEBS fibroblasts were seeded in growth medium 199 plus FCS 10% at about 1x105 cells/well in 24-well cluster plates. Ideally the cells should cover 80-90% of the well surface at the time of assay. TGFβ receptor assay was performed as described by Massagué (1987a). Briefly, the monolayers were washed with 1 ml/well binding buffer (MEM + 0,1% BSA + 25 mM Hepes pH 7,4) and incubated in this medium for 2 h at 37°C to allow dissociation of bound endogenous TGFβ. Then, cells were washed once more with 1 ml/well of binding buffer and 200 μl of ^{125}I-labeled TGFβ_1 was added over a concentration range of 1-500 pM.

Non specific binding was determined in the presence of 10 nM unlabelled TGFβ_1. Binding buffer alone was added to the four wells used for cell counts. Cells were incubated for 3 h at 4°C and the plates were gently agitated. The cell monolayers were then washed four times with ice cold Hanks' buffered saline (GIBCO) containing 0.1% BSA, 1% Triton X100, 10% glycerol, 20 mM Hepes, pH 7.4 and incubated for 40 min at 4°C.

Specific binding was determined by subtracting the non-specific from the total TGFβ_1 bound, the receptor affinity (Massagué, 1987b) and the number of receptors/cell were determined with Ligand software (Munson and Rodbard, 1980).

^{125}I-TGFβ receptors were analysed by 5.5% SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol, followed by autoradiography to detect affinity-labelled products (Massagué, 1987b).

RNA extraction and RNA Northern Blot analysis

Total RNA was isolated from 7 and 14 day chick embryo fibroblasts maintained for 4 h in MEM without serum, according to Chomczynski and Sacchi (1987). For RNA Northern blot analysis, equal amounts of total RNA (20 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred onto nylon membranes (Hybond N, Amersham) according to the manufacturer's instructions.

The blots were hybridised overnight at 42°C with ^{32}P-labelled cDNA probes and then subjected to autoradiography. The same filters were stripped and rehybridised with 1.3 kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA (pRGAPDH) for normalisation purposes. Differences in the intensity of the specific hybridisation bands from the autoradiograms were evaluated by computerised scanning densitometry.

cDNA probes

cDNA probes were constructed by polymerase chain reaction (PCR) amplification using primers designed according to the published gene sequences of TGFβ_3 and TGFβ_4 (Gen Bank accession n. M31154 and X08012 respectively). First, total RNA extracted from 7 and 14 day CEBS fibroblasts was reverse transcripted, and a fraction of the cDNA (1 μl) was amplified by PCR using primers directed against the mature polypeptide coding sequence for both TGFβ_3 and TGFβ_4. The primers sequences for chick TGFβ_3 and TGFβ_4 were: TGFβ_3 5' primer, 5'-CAA CTA CTG CTT CCG GAA CCT-3' (complementary to nd 951-971); TGFβ_3 3' primer, 5'-TTT CAC CAC CAT ATT GGA GA-3' (complementary to nd 1241-1260); TGFβ_4 5' primer, 5'-GAC CTC GAC ACC GAC TAC TGC TT-3' (complementary to nd 811-833); TGFβ_4 3' primer, 5'-ACT TGC AGG CAC GGA CCA CCA TA-

3' (complementary to nd 1125-1147). Amplified DNA products were analysed on 1.5% (wt/vol.) agarose gels containing ethidium bromide. The expected amplified product size for TGFβ_3 was 310bp, and for TGFβ_4 336bp. To confirm the authenticity of PCR products, the 310bp TGFβ_3 DNA product and the 336bp TGFβ_4 product were subjected to restriction enzyme mapping with specific restriction enzymes. The PCR products were further sub-cloned to a pGEM11Zf(+) vector (Promega).

Protein determination

Protein concentration was determined by Lowry *et al.* assay (1951) on aliquots of cell lysate.

Statistical analysis

Statistical analysis was performed using Student's *t*-test. Data were expressed as the mean±SD for quadruplicate cultures of a typical experiment. Similar results were obtained in three independent experiments performed for each point.

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