Int. J. Dev. Biol. 43: 059-067 (1999)

Original Article

Expression of N-cadherin, N-CAM, fibronectin and tenascin is stimulated by TGF-β1, β2, β3 and β5 during the formation of precartilage condensations

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ABSTRACT Cell surface adhesion and extracellular matrix proteins are known to play a key role in the formation of cell condensations during skeletal development, and their formation is crucial for the expression of cartilage-specific genes. However, little is known about the relationship between adhesion molecules (N-cadherin and N-CAM), extracellular matrix proteins (fibronectin and tenascin) and TGF-β1, TGF-β2 and TGF-β3 during *in vitro* precartilage condensations in mouse chondrogenesis. On these bases, we determined the participation of mammalian TGF-β1, TGF-β2 and TFG-β3 and *Xenopus* TGF-β5 on the expression of cell surface adhesion and extracellular matrix proteins during the formation of precartilage condensations. Also, we characterized the effects of TGF-βs on proteoglycan metabolism at different cellular densities in mouse embryonic limb bud mesenchymal cells. In TGF-β1 and TGF-β5-treated cultures, proteoglycan biosynthesis was higher than in controls, while there were no differences in proteoglycan catabolism, which caused the accumulation of cartilage extracellular matrix. When mesenchymal cells were seeded at three different cellular densities in the presence of TGF-βs, only high density cultures presented increased stimulation of proteoglycan biosynthesis, compared to low and intermediate densities. To determine whether the effect of TGF-βs on precartilage condensations is mediated through the expression of N-cadherin, N-CAM, fibronectin and tenascin, we evaluated their expression. Results showed that TGF-β1, TGF-β2, TGF-β3, and TGF-β5 differentially enhanced the expression of N-cadherin, N-CAM, fibronectin and tenascin in precartilage condensations, suggesting that TGF-β isoforms play an important role in the establishment of cell-cell and cell-extracellular matrix interactions during precartilage condensations.

KEY WORDS: *precartilage condensations, chondrogenesis, cell adhesion, ECM, TGF-β*

Introduction

The members of the TGF-β superfamily consist of several growth factors that share structural homology and similar biological activities in a wide variety of organisms from insects to humans (Massagué, 1990). Based on their structural and functional similarities, the members of the TGF-β superfamily have been subdivided into four groups which include: a) the TGF-β family with five members (TGF-β1-5); b) the activin/inhibin family; c) the BMP/Dpp/Vg-1 family and d) the Müllerian Inhibiting Substance (MIS) family (Massagué, 1990). Homology between TGF-β family isoforms is about 70% among mammalian species. Homology of chick TGF-β4 and frog TGF-β5 with human TGF-β1 is 82% and 76% respectively, with human TGF-β2 it is 64% and 66%, and with human TGF-β3 it is 71% and 69%, suggesting that TGF-β4 and TGF-β5 are the chick and frog homologs of mammalian TGF-β1 (Kondaiah *et al.*, 1990; Burt and Paton, 1992).

Among the diverse roles of TGF-β isoforms on cell differentiation are those related to cartilage differentiation. TGF-β isoforms have been shown to have stimulatory or inhibitory effects on cartilage cells *in vitro*, depending on the developmental origin and stage of these cells. Micromass cultures of embryonic limb bud mesenchymal cells have been used to study the sequence of events of chondrogenesis *in vitro*, since mesenchymal cells are able to aggregate, resembling *in vivo* condensation. Also, several growth factors when added exogenously to micromass cultures of

Abbreviations used in this paper: TGF-β, transforming growth factor beta; N-CAM, neural cell adhesion molecule; FBS, fetal bovine serum.

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0214-6282/99/$10.00

Printed in Spain
www.lg.ehu.es/ijdb

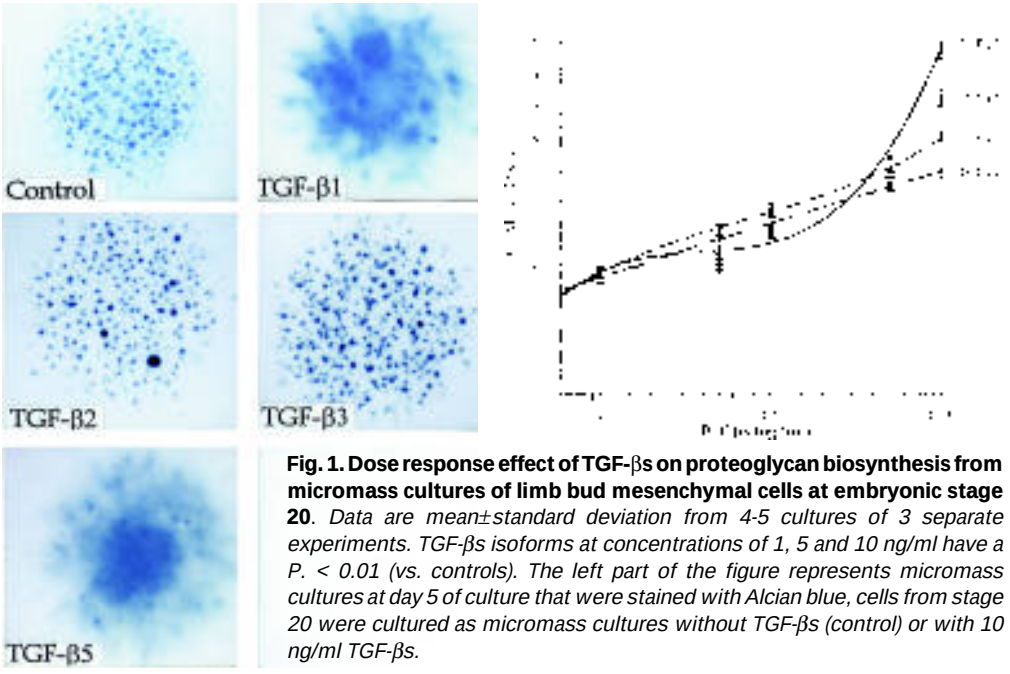

Fig. 1. Dose response effect of TGF-βs on proteoglycan biosynthesis from micromass cultures of limb bud mesenchymal cells at embryonic stage 20. *Data are mean±standard deviation from 4-5 cultures of 3 separate experiments. TGF-βs isoforms at concentrations of 1, 5 and 10 ng/ml have a P. < 0.01 (vs. controls). The left part of the figure represents micromass cultures at day 5 of culture that were stained with Alcian blue, cells from stage 20 were cultured as micromass cultures without TGF-βs (control) or with 10 ng/ml TGF-βs.*

chick limb mesenchymal cells elicit different responses. Exposure of chick mesenchymal cells of embryonic forelimbs of stages 23/24 and 22/23 to TGF-β1, TGF-β2 and TGF-β3 promotes chondrogenesis. Their role appears to be most effective when added during the first 2 days of culture, which is when mesenchymal cells are in the process of cellular condensation (Kulyk *et al.*, 1989; Carrington and Reddi, 1990; Leonard *et al.*, 1991; Roark and Greer, 1994), or during continuous exposure to TGF-β1 and TGF-β2 of distal subridge mesenchymal cells of stage 22/23 and of mesenchyme of stage 24 (Schofield and Wolpert, 1990; Tsonis *et al.*, 1994). Under other culture conditions TGF-β inhibits chondrogenesis; for example, in the presence of 0.5% serum, TGF-β1 and TGF-β2 depressed chondrocyte differentiation at embryonic stage 24/25, but that inhibition was overcome by BMP-3 and BMP-4 (Carrington *et al.*, 1991; Chen *et al.*, 1991). Moreover, exposure to TGF-β1 and TGF-β2 in the presence of 5% serum at 4-7 or 3 and 4 days of culture, reduced alkaline phosphatase activity and incorporation of ^{35}S-sulfate into mesenchymal cells of stage 23/24, but when TGF-β1 was applied during days 1-2, it caused an increase in both parameters (Carrington and Reddi, 1990). Other members of the TGF-β superfamily with chondroinductive capacity on limb bud mesenchymal cells *in vitro* are inhibin and activin (Chen *et al.*, 1993; Jiang *et al.*, 1993). These reports showed that TGF-β family members are most effective when added during the first two days, independently of culture conditions.

Cell-cell and cell-extracellular matrix (ECM) interactions are events that take place between interacting cells for chondrogenesis to occur. N-cadherin and N-CAM are cell adhesion molecules, whereas fibronectin and tenascin are ECM proteins involved in precartilage condensations (Hall and Miyake, 1995). The expression of N-cadherin, N-CAM and tenascin during limb development is restricted to precartilage condensations, whereas fibronectin is expressed in all limb bud mesenchyme and gradually enriched cell condensations (Ide, 1996). Perturbation of N-cadherin and N-CAM with neutralizing antibodies suppresses cartilage differentiation both *in vivo* and *in vitro* (Widelytz *et al.*, 1993; Oberlender and Tuan, 1994). The participation of fibronectin in precartilage condensation was evident *in vitro*, when an antibody specific for the exon IIIA of fibronectin (Gehris *et al.*, 1997) or when an antibody directed against the 29 kDa amino-terminal heparin-binding domain of fibronectin and an oligopeptide gly-arg-gly, which is a repeated motif in that fibronectin domain, blocked cartilage differentiation by inhibition of precartilage condensations (Frenz *et al.*, 1989a,b).

The expression of N-cadherin, N-CAM, tenascin and fibronectin during chick chondrogenesis by limb bud mesenchymal cells *in vitro* is up-regulated by growth factors such as TGF-β1 and activin (Leonard *et al.*, 1991; Jiang *et al.*, 1993; Tsonis *et al.*, 1994; Downie and Newman 1994, 1995). The formation of precartilage condensations during micromass cultures is evident at day 2 of culture giving rise to cartilage nodules. In control cultures, the pattern formation of cartilage is nodular, whereas in TGF-β-treated cultures, cellular condensations are broad and flat, giving rise to a continuous sheet of cartilage cells. As mentioned above N-cadherin, N-CAM, fibronectin and tenascin participate in the formation of precartilage condensations during chick chondrocyte differentiation (Mackie *et al.*, 1987; Frenz *et al.*, 1989a,b; Widelytz *et al.*, 1993; Oberlender and Tuan 1994). However, to our knowledge there is no available information on the expression of N-cadherin, N-CAM, fibronectin and tenascin in mouse limb mesenchymal cells and their regulation by TGF-β1, TGF-β2 and TGF-β3.

Recently, in our laboratory we observed that a transient exposure of mouse limb mesenchymal cells to TGF-β1, TGF-β2, TGF-β3 and TGF-β5 for 24 h at days 1 and 2 of culture, stimulates chondrogenesis, indicating that TGF-β isoforms may regulate chondrogenesis at early stages of chondrocyte differentiation (Chimal-Monroy and Díaz de León, 1997). In the present study we examined the action of three mammalian TGF-β isoforms and *Xenopus* TGF-β5 on proteoglycan metabolism at different cellular densities and on expression of specific proteins of precartilage condensations in mouse embryonic limb bud mesenchymal cells.

We found that the accumulation of cartilage extracellular matrix was due to a higher proteoglycan biosynthesis rather than low proteoglycan catabolism. In addition, when mesenchymal cells were cultured at three different cellular densities in the presence of TGF-β isoforms, only high density cultures presented greater stimulation of proteoglycan biosynthesis compared to low and intermediate densities. Moreover, TGF-β isoforms enhanced the formation of precartilage condensations through stimulation of the expression of cell adhesion (N-cadherin and N-CAM), as well as extracellular matrix (ECM) molecules (tenascin and fibronectin). These results suggest that TGF-β isoforms promote

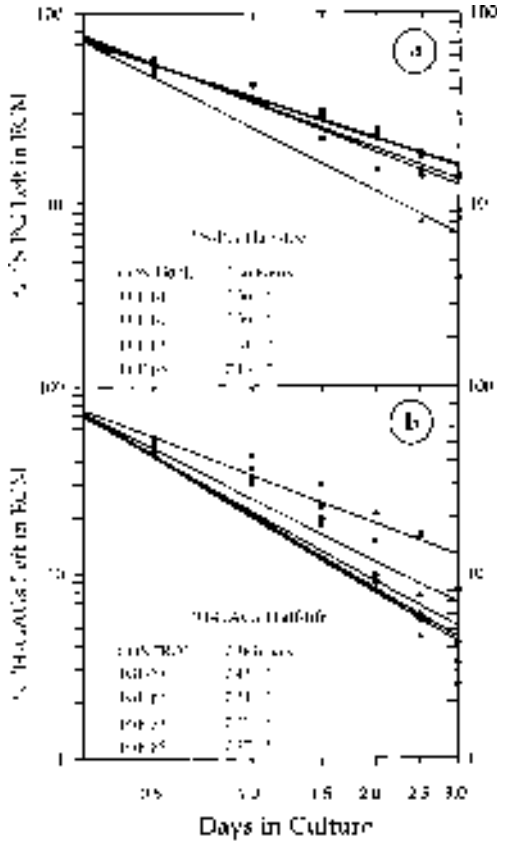

Fig. 2. Effect of TGF-βs on proteoglycan catabolism in stage 20 mouse limb bud mesenchymal cells. (a). *Catabolism of ^{35}S-sulfate radiolabeled proteoglycans and* **(b)** *catabolism of ^{3}H-glucosamine radiolabeled proteoglycans. Micromass cultures were pulsed 24 h with ^{35}S-sulfate and with ^{3}H-glucosamine in the absence of serum and TGF-βs were added at day 2 of culture. After this time, triplicate cultures were chased for the indicated times as indicated in the figure. Control (o); TGF-β1(•); TGF-β2(▲); TGF-β3(▼); TGF-β5(◆).*

mouse chondrocyte differentiation by enhancing the expression of molecules involved in the formation of precartilage condensations.

Results

Effect of TGF-β isoforms on proteoglycan metabolism

To test the effects of the TGF-β isoforms on cartilage extracellular matrix and proteoglycan metabolism, mesenchymal cells were seeded at high density in the presence of 2% FBS. They showed a dose-dependent response to TGF-β1, TGF-β2, TGF-β3 and TGF-β5, at day 5 of culture (Fig. 1). At this time, cartilage extracellular matrix was determined by staining with Alcian blue. When cultures were treated with TGF-β1 and TGF-β5 an entire cell layer of chondrogenic cells was observed, whereas control cultures, TGF-β2 and TGF-β3 showed a nodular pattern (Fig. 1). Cartilage matrix biosynthesis was measured by incorporation of ^{35}S-sulfate onto sulfated proteoglycans (Fig. 1). The maximal response of micromass cultures to TGF-β isoforms was obtained at a concentration of 10 ng/ml.

To determine whether the accumulation of cartilage matrix was due to lower proteoglycan catabolism or to increased proteoglycan biosynthesis, we determined the rate of proteoglycan catabolism in micromass cultures in response to TGF-β1, TGF-β2, TGF-β3 and TGF-β5 (Fig. 2). Catabolism of sulfated proteoglycans was very similar for all four TGF-β-treated micromass cultures compared to controls. Also, there were no differences in the half-lives of TGF-β-treated cultures and controls (Fig. 2a). The effects of TGF-β isoforms on ^{3}H-glucosamine GAG catabolism (Fig. 2b) were similar to those observed for sulfated proteoglycans (Fig. 2a).

Effect of TGF-β isoforms on cell cultures of low, intermediate and high density

We determined the capacity of TGF-β isoforms to stimulate biosynthesis of sulfated proteoglycans on culture conditions in which mesenchymal cells undergo minimal chondrogenic differentiation, i.e., low and intermediate densities and they were compared with high density cultures (Fig. 3). A dose of 10 ng/ml was selected to examine the time course of response to all four TGF-β isoforms, since this concentration elicited a maximal response at day 5 of culture in micromass cultures (Fig. 1). TGF-β isoforms were not able to enhance the synthesis of proteoglycans during day 1 and 3 of culture in the three cellular densities assayed.

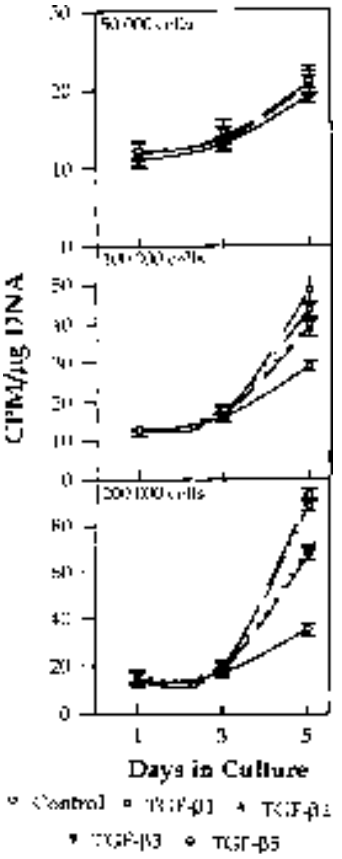

Fig. 3. Influence of TGF-βs on proteoglycan biosynthesis of low, intermediate and high density cultures from stage 20 mouse limb mesenchymal cells. *TGF-βs at concentration of 10 ng/ml were added to low, intermediate and high density cultures for 5 days. All cultures were incubated with ^{35}S-sulfate during 6 h at days 1, 3 and 5 of culture. Data represent means±standard deviation of 3 cultures of 2 separate experiments. P. < 0.01 (vs. controls).*

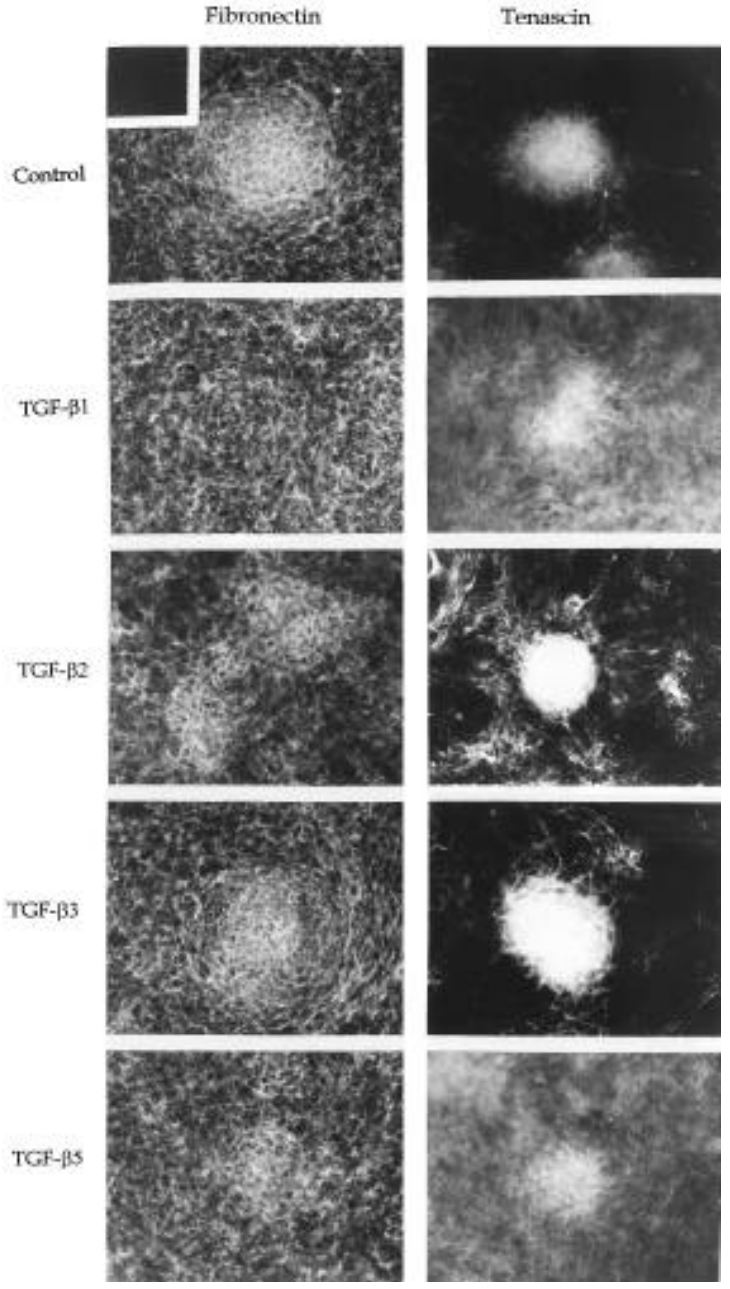

Fig. 4. Influence of TGF-βs on fibronectin and tenascin expression. *Indirect immunofluorescence was performed for fibronectin and tenascin in the presence of TGF-βs. Mesenchymal cells were cultured as micromass cultures without TGF-βs or with 10 ng/ml TGF-βs to determine the expression of fibronectin and tenascin.*

Nevertheless, at day 5 of culture, all TGF-β isoforms increased the synthesis of proteoglycans in high and intermediate cellular densities to higher levels than those obtained in controls. However, the greatest effect was obtained with high density cultures (Fig. 3).

Effect of TGF-β isoforms on the expression of extracellular matrix molecules

It has been shown that TGF-β isoforms promote chondrogenesis in mouse limb mesenchymal cells when added during the first two days of culture, suggesting that the action of TGF-β isoforms occurs at early stages of chondrogenesis (Chimal-Monroy and Díaz de León, 1997). In this set of experiments we evaluated the expression of molecules involved in the formation of precartilage condensations. The administration of TGF-β isoforms was for two consecutive days. The staining distribution of precartilage nodules with anti-fibronectin was around cells forming a network of fibronectin-rich fibrils in the cell condensations. The effect of TGF-β1, TGF-β2, TGF-β3 and TGF-β5 treatment on fibronectin distribution was more pronounced than in control cultures. Under these conditions, fibronectin was diffusely organized around cells and concentrated in cell condensations (Fig. 4). For tenascin, its distribution was different in relation to the fibronectin distribution presented in both control and TGF-β-treated cultures (Fig. 4). In control and TGF-β treated cultures, it was organized around cells in precartilage condensations, but the staining was stronger in TGF-β-treated cultures (Fig. 4). By Western-blot analysis we determined that the expression of fibronectin and tenascin was higher in TGF-β-treated than in control cultures (Fig. 5). By densitometric analysis we found that fibronectin was enhanced by TGF-β1 in 1.97-, TGF-β2 in 1.35-, TGF-β3 in 1.42-, and TGF-β5 in 1.89-fold compared to controls. Tenascin was enhanced by TGF-β1 in 2.80-, TGF-β2 in 2.05-, TGF-β3 in 2.15- and TGF-β5 in 2.3-fold with respect to controls (Fig. 5). Similar results were obtained when TGF-β isoforms were applied by a pulse of 24 h at day 1 of culture or by a pulse of 24 h at day 2 of culture (data not shown).

Effect of TGF-β isoforms on the expression of cell adhesion molecules

We evaluated the expression of cell adhesion molecules in response to TGF-β isoforms when it was added for two consecutive days. The expression of N-cadherin and N-CAM, in control cultures, was evident in the precartilage condensations (Fig. 6). The staining of nodules with anti-N-cadherin was localized in cellular aggregates around cells; in contrast, a rather diffuse staining with anti-N-CAM was observed (Fig. 6). All four TGF-β isoforms increased the expression of N-CAM and N-cadherin (Fig. 6). The most potent TGF-β isoform in the stimulation of precartilage aggregates was TGF-β1. Also, this observation was confirmed by Western-blot analysis (Fig. 7). N-cadherin expression was stimulated by TGF-β1 in 2.60-, TGF-β2 in 1.40-, TGF-β3 in 1.70- and TGF-β5 in 2.10-fold in relation to control cultures; N-CAM expression was stimulated by TGF-β1 in 2.10-, TGF-β2 in 1.35-, TGF-β3 in 1.70- and TGF-β5 in 1.85-fold. (Fig. 7). Similar results were found when TGF-β isoforms were applied by a pulse of 24 h at day 1 of culture or by a pulse of 24 h at day 2 of culture (data not shown).

Discussion

In the present study we demonstrate that TGF-β1, TGF-β2, TGF-β3 and TGF-β5 enhance chondrogenesis in micromass cultures of embryonic mouse cells of stage 20 in the presence of 2% serum. Similar patterns have been obtained in chick and mouse mesenchymal cells upon treatment with TGF-β1, TGF-β2, TGF-β3, BMP-2, 3 and 4, activin and inhibin (Kulyk *et al.*, 1989; Carrington and Reddi, 1990; Leonard *et al.*, 1991; Roark and Greer, 1994; Chimal-Monroy *et al.*, 1996; Chimal-Monroy and

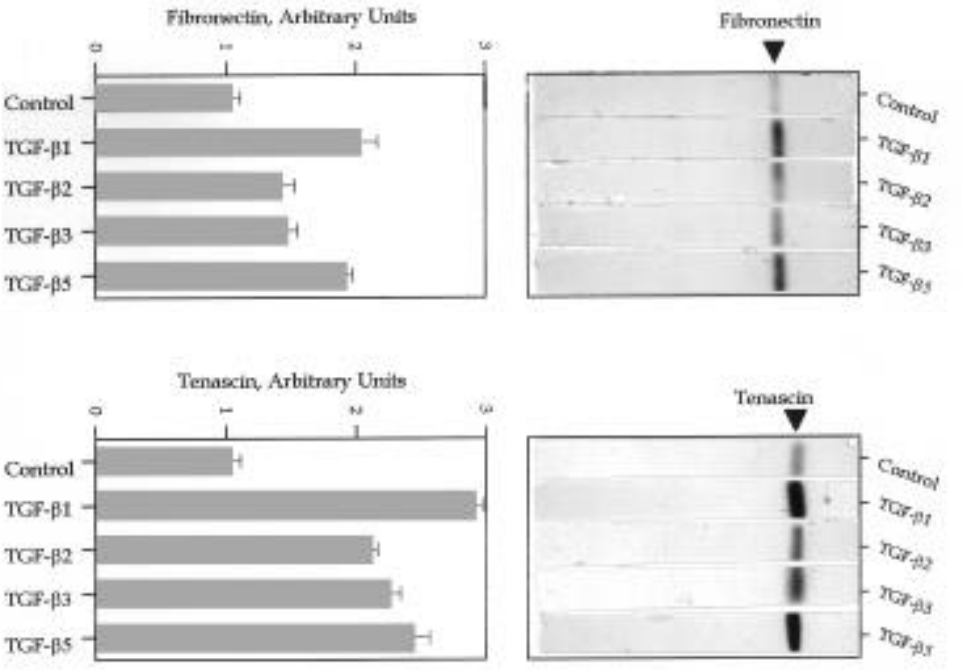

Fig. 5. Western blot analysis of fibronectin and tenascin. *Immunoblot analysis of fibronectin and tenascin was obtained from control and TGF-βs treated cultures at day 2 of culture. Protein load was 40 µg/lane in SDS-PAGE. The expression of fibronectin and tenascin was used as ECM protein markers of precartilage condensations and is given in arbitrary units, as measured by scanning densitometry of Western blots.*

Diaz de León, 1997) suggesting that TGF-β5, as other members of the TGF-β superfamily, could play an important role during cartilage differentiation.

To determine whether the formation of a continuous sheet of cartilage cells in TGF-β1 and TGF-β5-treated cultures was a consequence of higher proteoglycan biosynthesis or of lower proteoglycan catabolism, we evaluated the effect of these growth factors on the rate of catabolism of radiolabeled ^{35}S-sulfate and ^{3}H-glucosamine material and compared it with the effect of TGF-β2 and TGF-β3. Results show that proteoglycan catabolism with all four TGF-β isoforms is similar to controls. This suggests that TGF-βs may play a physiological role in the maintenance of chondrocyte homeostasis of proteoglycan metabolism. We find no differences between our results and those obtained by Kulyk *et al.* (1989) with TGF-β1 in chick limb bud mesenchymal cells, because they do not report effects of TGF-β1 on the rate of ^{35}S-sulfate-material catabolism.

On the other hand, TGF-β isoforms were able to stimulate cartilage differentiation, by mouse limb mesenchymal cells cultured at high and intermediate densities, when assayed in the presence of 10 ng/ml TGF-βs. At days 1 and 3 of culture at high and intermediate densities TGF-β isoforms did not enhance proteoglycan production. The effect on proteoglycan biosynthesis was observed at later times of culture. However, the effect was stronger in high density cultures than in low and intermediate densities. It is important to mention that, in contrast to other members of the TGF-β superfamily, all four TGF-β isoforms assayed in the present study were not able to stimulate cartilage formation under cellular conditions in which cartilage formation does not normally occur (Carrington and Reddi, 1990). This suggests that TGF-β1, TGF-β2, TGF-β3 and TGF-β5 are growth factors which enhance cartilage differentiation only under culture conditions in which cartilage formation is favored. TGF-β5 itself is not expressed in mammals and hence is not a candidate of mouse chondrogenesis endogenous regulation. However, for its homology with TGF-β1, the functional effects of TGF-β5 on cartilage differentiation could mimic the action of TGF-β1 endogenously expressed in mesenchyme itself.

Formation of precartilage condensations is a critical event for cartilage differentiation, in which cell-cell and cell-ECM interactions take place. At day 2 of culture under in micromass culture conditions, cellular condensations that give rise to cartilage nodules have been formed. At this time, the morphology of condensations of control cultures is nodular, whereas in TGF-β-treated cultures, they are broad and flat, originating a continuous sheet of cartilage cells.

Under micromass culture conditions, the formation of precartilage condensations, in response to growth factors, may be regulated by an increase in cell number or by recruitment of cells into the chondrogenic differentiating tissue (Jiang *et al.*, 1993; Hall and Miyake, 1995). In a previous study, we demonstrated that in TGF-β1 and TGF-β5-treated mouse mesenchymal cells during days in which the formation of precartilage condensations occurs, cell proliferation was not affected. TGF-β2 increased this activity at day 3 of culture, when the formation of precartilage condensations ends, but at day one, when the formation of precartilage condensations begins, there were no differences in treated culture as compared to controls. Finally, TGF-β3 showed a decrease in cell proliferation in relation to controls (Chimal-Monroy and Díaz de León, 1997). These results together with those obtained in the present study, suggest that TGF-β isoforms may regulate mesenchymal condensations by recruitment of cells into precartilage condensations. This recruitment may be mediated by an increase in the expression of N-cadherin, N-CAM, tenascin and fibronectin allowing cell-cell and cell-ECM interactions. As a consequence of this cellular recruitment, associated with an enhanced expression of cell surface adhesion and ECM proteins, precartilage condensations continue their differentiation towards

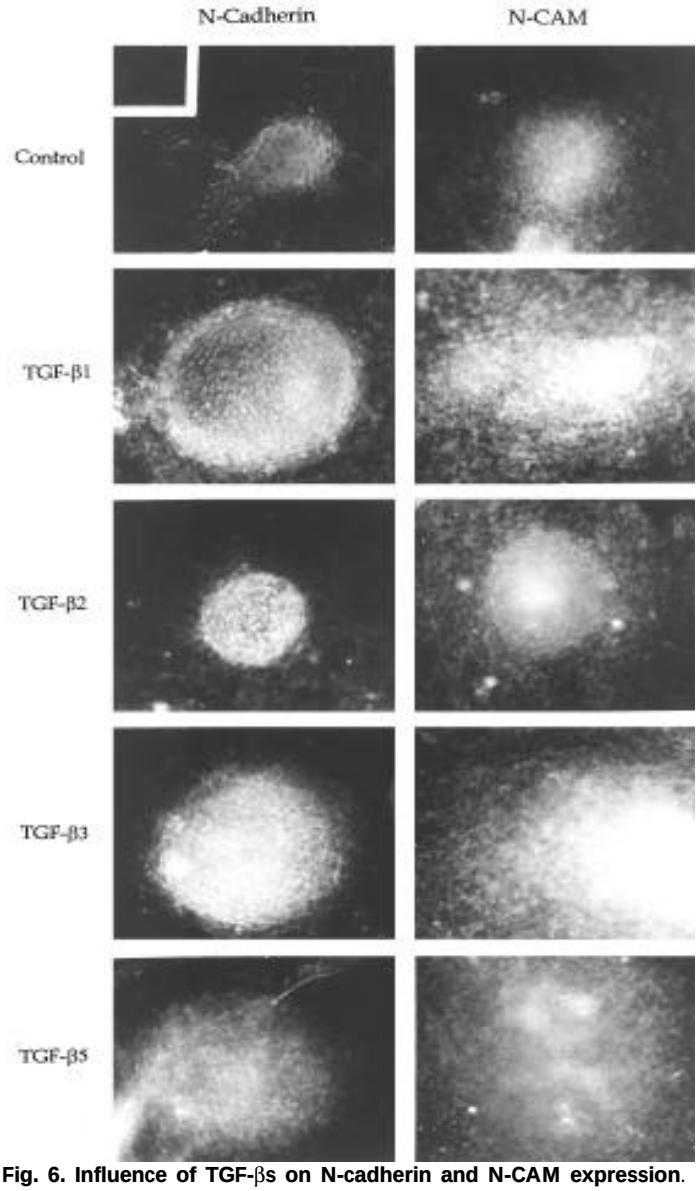

Fig. 6. Influence of TGF-βs on N-cadherin and N-CAM expression. *Indirect immunofluorescence was performed for N-cadherin and N-CAM at day 2 of culture in the presence of TGF-βs. Mesenchymal cells were cultured as micromass cultures without TGF-βs or with 10 ng/ml TGF-βs to determine the expression of N-cadherin and N-CAM.*

cartilage, increasing the expression of cartilage-specific genes. It is well known that during chondrogenesis, formation of cellular aggregates involves active participation of N-CAM, N-cadherin, tenascin and fibronectin (Mackie *et al.*, 1987; Frenz *et al.*, 1989a,b; Widelytz *et al.*, 1993; Oberlender and Tuan 1994; Gehris *et al.*, 1997).

In the chick limb bud mesenchymal cells TGF-β1 increases the expression of N-cadherin and fibronectin (Leonard *et al.*, 1991; Tsonis *et al.*, 1994). Also, activin stimulates the expression of N-CAM and tenascin (Jiang *et al.*, 1993). However, to our knowledge there is no available information suggesting that the expression of N-cadherin, N-CAM, fibronectin and tenascin is stimulated by TGF-β1 or other TGF-β isoforms in the mouse model. The present study demonstrates for the first time that TGF-β isoforms regulate the formation of precartilage condensations mediated by the expression of N-CAM, N-cadherin, fibronectin and tenascin in mouse limb mesenchyme. Downstream effects of TGF-β on the expression of these extracellular matrix and cell-cell adhesion proteins have been demonstrated in several systems. (Massagué, 1990 and references therein).

Using the mouse model, the present study shows that the distinct molecules were stimulated by the TGF-βs to different levels compared to controls. To illustrate this, Figure 8 groups the results presented in Figures 5 and 7 according to each growth factor. It is evident that TGF-β1 stimulates the expression of N-cadherin, N-CAM, tenascin and fibronectin to higher levels than the other TGF-βs. We also found that tenascin is stimulated in higher proportion than other molecules, followed by N-cadherin, fibronectin and N-CAM. This suggest that TGF-β isoforms stimulate differentially the expression of molecules involved in the establishment of cell-cell and cell-ECM interactions during formation of precartilage condensations. Also, these growth factors may function cooperatively, by which one TGF-β isoform may reinforce or substitute the action of the other TGF-β isoform during *in vitro* aggregation of precartilage cells. The enhancement of these molecules on cellular aggregates by each TGF-β, is similar when the administration was consecutive for two days or when it consisted of a 24 h pulse at days 1 or 2 of culture.

The expression of these molecules, as occurs in precartilage condensations, may be a general event for all skeletal elements (Hall and Miyake, 1995). However, it is important to keep in mind that the induction of mesenchymal cells towards chondrogenic lineage may be executed by bone morphogenetic proteins (BMPs; Kingsley *et al.*, 1992) and growth and differentiation factors (GDFs; Storm *et al.*, 1994), depending of the skeletal element, and perhaps TGF-β members, and other growth factors in response to BMPs and GDFs, could enhance and maintain the formation of precartilage condensations.

Materials and Methods

Cell culture

Embryos from Balb/c mice were used in this study and staged according to Theiler (1972). Mesenchymal cells were obtained by dissociating forelimb buds of stage 20 according to Ahrens *et al.* (1977). Cellular suspension was adjusted at three different densities (low $0.5x10^7$ cells/ml; intermediate $1x10^7$ cells/ml and high density, $2x10^7$ cells/ml) in DMEM supplemented with 2% fetal bovine serum (FBS). Growth factors (R&D Systems) were added to the medium at concentrations of 0.1, 0.5, 1, 5 and 10 ng/ml.

Alcian blue staining

To obtain a quantitative measurement of differentiated cartilage matrix, 5-day cultures were stained overnight with 1% Alcian blue in 3% acetic acid and bound stain was extracted with 0.3 ml 4.0 M guanidine hydrochloride and quantitated using an ELISA reader at a wavelength of 600 nm.

DNA quantitation

The method of Labarca and Paigen (1980) was used to determine the amount of DNA in micromass cultures. The assay was performed using duplicate 50 µl aliquots of each sample in a total volume of 4 ml of reaction mixture. The resulting fluorescence was measured and the quantity of DNA was determined by comparison with a standard curve.

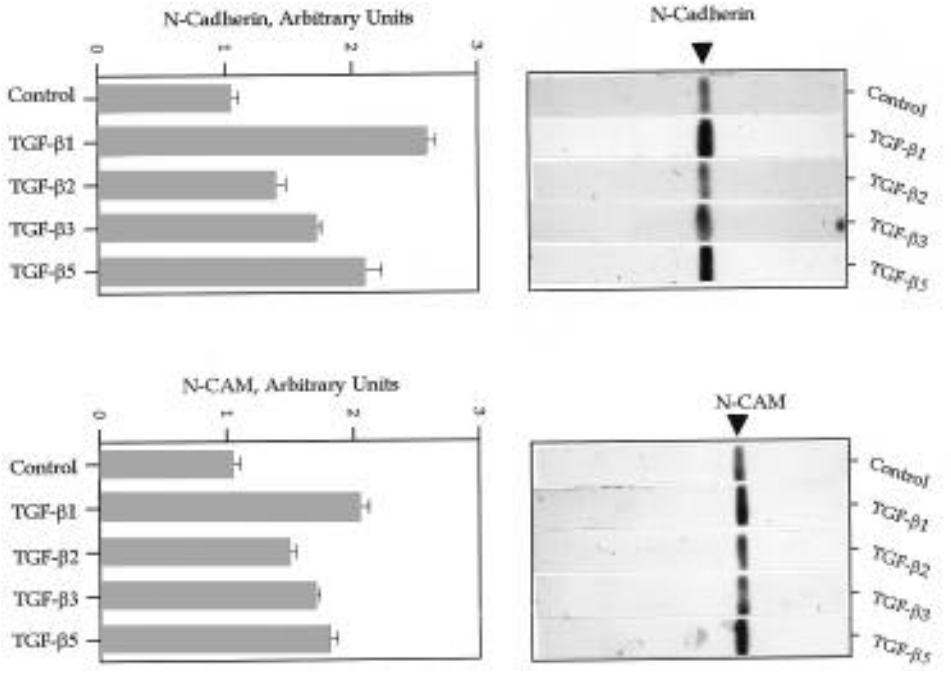

Fig. 7. Western-blot analysis of N-cadherin and N-CAM. *Immunoblot analysis of N-cadherin and N-CAM was obtained from control and TGF-βs treated cultures at day 2 of culture. Protein load was 40 μg/lane in SDS-PAGE. The expression of N-cadherin and N-CAM was used as cell adhesion protein markers of precartilage condensations and is given in arbitrary units, as measured by scanning densitometry of Western blots.*

Biosynthesis of glycosaminoglycans

Synthesis of sulfated glycosaminoglycans by the cultures was evaluated by ^{35}S-sulfate incorporation. Cells were labeled with 5 μCi/ml ^{35}S-sulfate (carrier free; Amersham, UK) at days 1, 3 and 5 of culture for 6 h in serum-free medium and in the presence of growth factors. Cells were resuspended in 50 mM Tris-HCl, pH 8.0, with 3% ethanol and digested with pronase (1 mg/ml; Sigma) at 50°C for 48 h. This material was centrifuged for 20 min at 13 000xg, and the supernatant was adjusted to a final 3N NaOH. Thereafter, it was precipitated with 5% TCA overnight at 4°C. After centrifugation for 20 min at 13000xg the supernatant was dialyzed against distilled water. Glycosaminoglycans were precipitated with 3 volumes of 96% ethanol/1.3% potassium acetate (Oohira *et al.*, 1977). Aliquots were taken and ^{35}S- incorporated into sulfated GAGs was measured in a liquid scintillation counter (Beckman).

Catabolism of proteoglycans

To determine the rate of proteoglycan catabolism, cultures were maintained for 24 h without serum and without growth factors, then micromass cultures were incubated with 5 μCi/ml ^{35}S-sulfate and ^{3}H-glucosamine for 24 h. Cultures were exhaustively washed with PBS, and serum-free medium and medium with growth factors were added. During the experimental period the medium with and without growth factors was changed every 12 h and the discarded medium was collected. Every 12 h the content of ^{35}S-sulfate in the ECM was determined. Briefly, micromass cultures were washed five times with culture medium and together with the daily collected medium they were analyzed for ^{35}S-sulfate and ^{3}H-glucosamine content after digestion with pronase as described above. To determine the rate of catabolism, the data were fitted to a logarithmic curve and the half-life was calculated according to Schafer *et al.* (1993).

Immunofluorescence staining

Immunostaining for N-CAM, fibronectin and tenascin was performed as described elsewhere (Jiang *et al.*, 1993). Samples at day 2 of culture were fixed in 2.5% paraformaldehyde and incubated with either polyclonal anti-N-CAM (gift from Urs Rutishauser), anti-tenascin and anti-fibronectin (Chemicon). For N-cadherin immunostaining (monoclonal MNCD2, gift from Masatoshi Takeichi), cells were washed with Hepes-buffered salt solution, fixed with microwave oven for 5-30 seconds and with methanol at -20°C and incubated with MNCD2 (Matsunami and Takeichi, 1995). Cultures were developed with fluorescein-conjugated goat anti-rabbit or rabbit anti-rat secondary antibodies and visualized under an immunofluorescence microscope (Nikon). Controls were incubated without primary antibodies.

Western-blot analysis

To obtain N-cadherin, N-CAM, fibronectin and tenascin, cells after culture were treated with a lysis buffer consisting of 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% deoxycholate, 1% SDS, and 2 mM EDTA containing a cocktail of phosphatase inhibitors (2 mM PMSF, 0.2 TIU/ml aprotinin, 1 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor and 30 mM sodium phosphate) according to Lightner *et al.* (1994). Total protein extracts were mixed with electrophoresis sample buffer. Twenty five micrograms of total protein from each condition were loaded onto 7.5% SDS-PAGE for N-cadherin and N-CAM, fibronectin and tenascin onto 6.0% SDS-PAGE and electrophoresed for 1 h (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes (BIORAD; Burnette, 1981). The detection of the N-cadherin, N-CAM, tenascin and fibronectin was made with the antibodies mentioned above. The Western blot was performed with the Chemiluminiscence Western Blotting kit (Amersham Life Sciences).

Statistical analysis

Data are presented as the mean ± standard deviation. Comparisons of experimental groups with controls were carried out by one-way analysis of variance, and statistical differences between groups were assessed by Student's *t* test (SigmaStat Statistical Analysis System, Jandel Corporation). Significant differences were accepted at the *P.* <0.05 level.

Acknowledgments

The authors thank Dr. Masatoshi Takeichi for anti-N-cadherin gift and Dr. Urs Rutishauser for anti-N-CAM gift, MVZ. Georgina Diaz Herrera and MVZ Javier Arzate for their technical assistance and Mr. José Avilés for art work. Also, the authors thank Isabel Pérez Monfort for her English revision and Isabel Reyes Hernández for preparation of the manuscript. This study was supported by PAPIID grant IN215696 from Universidad Nacional Autónoma de México.

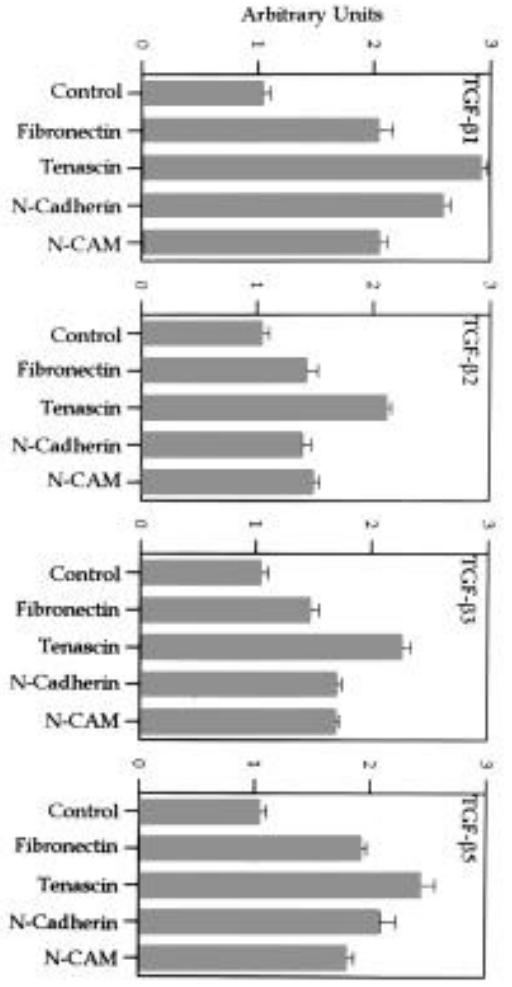

Fig. 8. Stimulation of N-cadherin, N-CAM, tenascin and fibronectin by TGF-β isoforms. *This figure groups the data presented in Figures 5 and 7 according to each growth factor. TGF-β1 stimulates the expression of N-cadherin, N-CAM, tenascin and fibronectin to higher levels than other TGF-βs.*

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Received: July 1998
Accepted for publication: September 1998