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The involvement of TGFβ1 in early avian development: gastrulation and chondrogenesis

E.J. Sanders, S. Prasad, N. Hu

Department of Physiology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7

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Abstract. We examined the effects of transforming growth factor-β1 (TGFβ1) and a neutralizing monoclonal antibody on two phases of early chick embryo development: gastrulation and chondrogenesis. We carried out experiments in vivo and in vitro on mesoderm cells from the gastrulating embryo at day 1, and on sclerotome cells from day 3 embryos, having previously shown that this factor is present among these cells at these stages of development. Addition of the antibody to cultures of these cells produced a dose-dependent decrease in cell outgrowth and spreading and concomitantly reduced fibronectin deposition. In vivo studies of the effects of TGFβ1 on mesoderm during gastrulation were carried out by grafting beads carrying this agent into gastrulating embryos. We used beads of ion-exchange resin as well as hydrolysed polyacrylamide, and found that the grafts produced an accumulation of mesoderm cells around the implant and, at later stages, the formation of enlarged somites. There was no effect on embryonic axis formation. Studies of bromodeoxyuridine (BrdU) incorporation indicated that the mesoderm accumulation was due, at least in part, to an increase in cell proliferation. However, examination of the effect of TGFβ1 on BrdU incorporation by mesoderm during gastrulation and sclerotome cells in vitro indicated in inhibition of cell proliferation, an inconsistency explained in terms of the variation between the in vivo and in vitro conditions. We conclude that TGFβ1 is both appropriately located, and is able, to influence cell proliferation among the mesodermal cell populations during early development, and that this effect contributes to the overall control of mesodermal morphogenesis. Chondrogenesis was studied in vitro using micromass cocultures of sclerotome cells with notochord on a permeable substratum. Under these conditions, the addition of TGFβ1 caused an increase in the deposition of Alcian blue-stainable material, indicating a stimulation of chondrogenesis. We suggest that this result, coupled with the previous demonstration that TGFβ1 is present among the sclerotome cells in the embryo at this time, supports the contention that this factor exerts a regulatory effect on sclerotome cell differentiation.

Key words: Chick embryo – Development – TGFβ – Gastrulation – Chondrogenesis

Correspondence to: E.J. Sanders

Introduction

Gastrulation in birds involves a co-ordinated sequence of cellular interaction, cell translocation and cell transformation, resulting in the formation of a new population of cells, the mesoderm, from the very early two-layered embryo (Sanders 1986, 1989). In this process, epithelial cells of the upper epiblast layer undergo morphogenetic movements and ingress through a mid-line structure, the primitive streak (Bellairs 1986), to emerge as fibroblastic mesenchyme cells. The latter then disperse in the tissue space and interact with the surrounding extracellular matrix and overlying basement membrane to differentiate into several mesoderm-derived cell populations (Bellairs et al. 1992).

The factors influencing this early cell transformation are unclear, but appear to involve a combination of cell-to-cell and cell-to-matrix interactions. The possible involvement of transforming growth factor-β in early developmental processes (Rizzino 1988; Nilsen-Hamilton 1990; Roberts et al. 1990) led to an earlier investigation (Sanders and Prasad 1991) in which it was shown that transforming growth factor-β1 (TGFβ1) could be detected immunocytochemically in the tissues of the gastrulating chick embryo. It was also demonstrated that this factor could effect a phenotypic transformation of embryonic cells in vitro and influence their deposition of extracellular matrix when cultured on various substrata. These results provided circumstantial evidence for an in vivo role for TGFβ1 in the cellular interactions and transformations of early chick development. In an effort to substantiate this conclusion, we have now ex-

tended these studies by examining the effects of a TGFβ-neutralizing antibody on the in vitro phenomena described above. We have also grafted beads soaked in TGFβ1 and its antibody into embryos, in order to study in vivo effects directly. Previous experiments have shown that TGFβ1 is able to modulate the proliferative rates of several cell types (Barnard et al. 1990; Massagué 1990; Moses et al. 1990), although whether the effect is stimulatory or inhibitory may depend on the cell involved and on the experimental conditions. Results presented here are consistent with that conclusion.

Subsequent to gastrulation, TGFβ1 has been localized in several embryonic tissues of the chick, including the sclerotome (Sanders 1992). Sclerotome cells are destined to differentiate into chondrocytes (Hall 1977), and because TGFβ1 has been shown to be important in the initiation and/or modulation of chondrogenesis in other cell systems (Seyedin et al. 1988; Kulyk et al. 1989; Carrington and Reddi 1990; Joyce et al. 1990; Leonard et al. 1991), we have used micromass co-cultures of sclerotome cells with notochord in order to investigate the possible role of TGFβ1 in regulating the chondrogenic potential of these cells.

Materials and methods

White Leghorn hen's eggs were incubated for either 24 h or 3 days to bring them to either stage 5 or stage 18 of Hamburger and Hamilton (1951).

Cell culture. Tissues from the gastrulating embryo were dissected and cultured as described previously (Sanders and Prasad 1991). Briefly, pieces of mesoderm and sclerotome tissue, approximately 100 μm in diameter, were dissected from embryos at stages 5 and 18 respectively, and cultured on fibronectin- or laminin-coated coverslips in medium 199 containing 10% fetal bovine serum and antibiotics. A functionally-neutralizing monoclonal antibody to TGFβ1 and β2 (1D11.16; Celtrix Laboratories; Dasch et al. 1989) was added to the culture medium at various concentrations between 10 and 100 μg/ml, and the cultures were incubated at 37° C in a humidified CO_2 incubator for the required length of time. Controls were carried out in which an irrelevant mouse IgG was substituted for the antibody, and reversibility was tested by replacing the antibody with normal medium after antibody treatment. After incubation, explants were scored for size by counting the cell diameters surrounding the central residual tissue mass, and for fibronectin deposition using immunofluorescence technique as described previously (Sanders and Prasad 1991).

Embryo culture. Embryos at stage 5 of development were cultured according to the method of New (1955) by explanting the embryos, ventral surface uppermost, on their vitelline membrane stretched over a glass ring of 25 mm internal diameter. The embryo was cleaned of adhering yolk, using Pannett and Compton's saline, and incubated at 37° C for 1 h in order to allow the embryo to spread fully on its membrane.

Ion exchange resin beads (Tickle et al. 1985) or chips of hydrolysed polyacrylamide (Hypa; Zackson and Steinberg 1989), that had previously been treated with TGFβ1 or its antibody, were implanted into the embryo, after reflecting a flap of endoderm from regions lateral to the primitive streak and about halfway along the length of the streak. The ion exchange resin beads (AG1-X2 in the chloride form or formate form, BioRad) and pieces of Hypa were soaked in TGFβ1 (100 ng/ml; R and D Systems) or the 1D11.16 antibody (0.1–0.2 mg/ml) overnight at 4° C prior to implanting. The smallest beads and pieces of Hypa were selected (approximately 75 μm diameter) and transferred using the tips of electrolytically sharpened tungsten needles. Both materials stuck firmly to the embryonic tissue and were not dislodged by subsequent growth of the embryo or by experimental manipulations. Controls were carried out using beads soaked in saline under identical conditions ($n=25$), and also using embryos that had been dissected only, without any bead implant ($n=10$).

After implanting beads, the embryos were returned to the incubator for 6 h before fixing with 4% formol saline and routinely processing for wax embedding. Sections from a total of 85 grafted embryos were cut at 8 μm and stained with haematoxylin and eosin for examination.

Assessment of cell proliferation in vivo and in vitro. Some embryos that had received a graft of beads were returned to the incubator for 1 h and then covered with approximately 0.2 ml of 5-bromo-2'-deoxy-uridine (BrdU, Sigma Chemical Co.) at a concentration of 10 μg/ml for 2 h. The embryos were rinsed with warm saline and returned to the incubator for a further 3 h before being fixed in 4% paraformaldehyde overnight and subsequently processed for immunocytochemical detection of incorporated BrdU. Paraffin-wax sections through the area containing the graft were dewaxed and rehydrated with decreasing concentrations of ethanol to 50%. The sections were quenched for endogenous peroxidase with 1% hydrogen peroxide in 50% methanol for 30 min, washed in phosphate-buffered saline (PBS) and incubated in 0.5% bovine serum albumin with 0.05% Tween 20 for 1 h. Sections were incubated in an undiluted monoclonal antibody to BrdU (Amersham) for 1 h and washed thoroughly with PBS. The antibody was detected by incubation with biotinylated goat anti-mouse IgG (1:50 in PBS) for 1 h, followed by the avidin-peroxidase method using an "ABC kit" (Vector Laboratories), and 0.05% diaminobenzidine with 0.03% hydrogen peroxide for 10 min. Control experiments were carried out using beads that had not been exposed to TGFβ1.

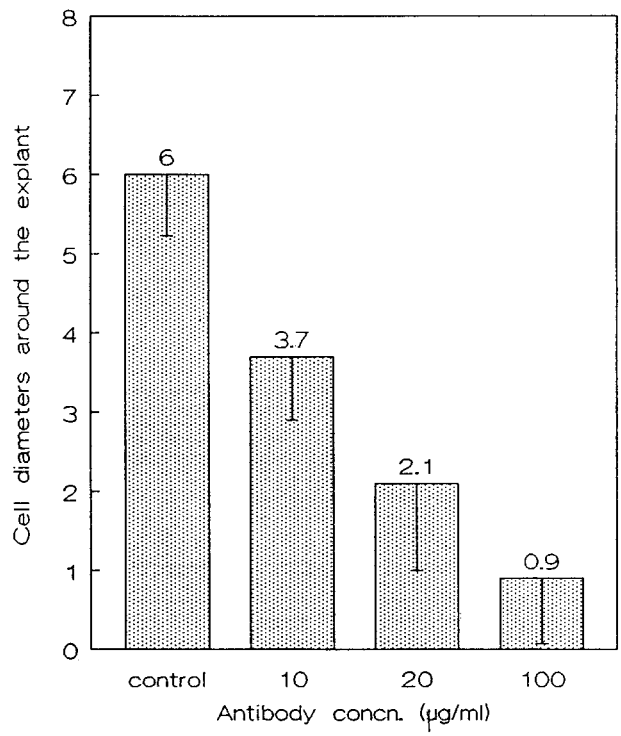


Fig. 1. Dose-dependent effect of 1D11.16 anti-TGFβ1 and β2 antibody on the outgrowth of sclerotome cells in culture. The explants were made in the presence of antibody and grown for 18 h before counting the number of diameters of spread cells around the central explant mass

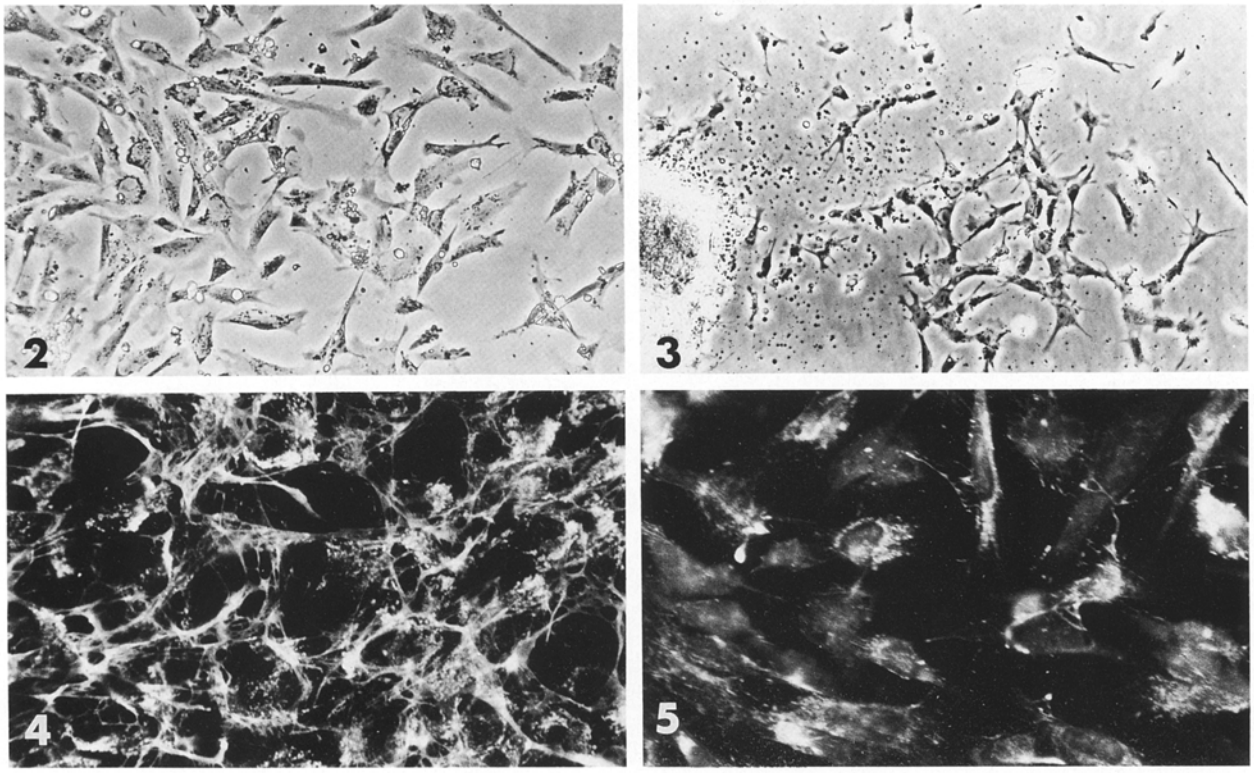


Fig. 2. Sclerotome cells cultured in normal medium for 3 days. The cells are well spread and fibroblastic in appearance. ×95

Fig. 3. Sclerotome cells cultured in medium containing antibody 1D11.16 (100 μg/ml) for 3 days. The cells are fewer in number and poorly spread, but the effect observed was reversible by replacing this medium with medium not containing antibody. Cells grown in medium containing non-specific mouse IgG appeared normal. ×95

Fig. 4. Sclerotome cells cultured in normal medium for 3 days. A network of fibronectin covers the explant. ×470

Fig. 5. Sclerotome cells cultured in medium containing antibody 1D11.16 (10 μg/ml) for 3 days. Relatively little fibronectin is deposited on the surface of the explant. ×470

Cell proliferation in cell cultures was assessed by culturing mesoderm from stage 5 embryos, or sclerotome from stage 18 embryos on fibronectin- or laminin-coated coverslips, or on uncoated coverslips, overnight in drops of culture medium as described above, in the presence or absence of TGBβ1 or 1D11.16 antibody. Next day, the cultures were topped up with culture medium containing 10 μg/ml BrdU and returned to the incubator for 2 h. After washing in Tyrode's saline, the cultures were fixed with 4% paraformaldehyde overnight. Incorporated BrdU was detected immunocytochemically as described above. Rates of cell proliferation were measured by counting a minimum of 1000 nuclei for each condition and determining the percentage of labelled nuclei. Only fully spread cells were counted. Each condition was repeated at least twice using a minimum of four individual cultures for each experiment.

Micromass cultures of sclerotome cells with notochord. In order to assess the chondrogenic potential of sclerotome tissue, cultures were set up according to methods modified from those of Cheney and Lash (1981) and of Paulsen and Solursh (1988). Sclerotome tissue was dissected from stage 18 embryos that had been treated with 0.1% trypsin for 45 min at 37° C. The trypsinization was designed to loosen tissues, but not to dissociate the cells. During dissection, tissues were stored in calcium- and magnesium-free (CMF) Tyrode's saline. The tissue was then incubated for 45 min at 37° C in 0.002 M ethylenediamine tetra-acetic acid (EDTA) in CMF Tyrode's saline, and dissociated into single cells by repeatedly pipetting, using a finely drawn out siliconized pipette. The cells were centrifuged and resuspended in culture medium. Cell concentration was then measured using a haemacytometer and adjusted to approximately 2.5×10^6 cells/ml of culture medium. Cells were cultured on 9-mm-diameter "Cyclopore" cell culture inserts (0.45 − μm pore size; Falcon, Becton Dickinson Labware) suspended in wells of a 24-well tissue culture plate (Linbro, Flow Laboratories). Cells were added to the inserts in a volume of 250 μl, and a similar volume was added to the outer well, so that the cells were exposed to medium on both sides of the permeable insert membrane. In order to induce chondrogenesis by the sclerotome cells, it was necessary to include pieces of notochord in the culture (Cheney and Lash 1981). In each culture, five pieces of notochord were included, each piece being approximately 200 μm in length. Cultures were incubated for 6 days at 37° C, with three or four changes of culture medium during this period. Experimental situations were set up with cultures containing TGFβ1 (50 or 100 ng/ml) or antibody 1D11.16 (0.2 mg/ml). Control cultures for these experiments contained either no TGFβ1, or mouse IgG respectively. After incubation for 6 days, the cultures were fixed for 30 min with 2% acetic acid in ethanol, washed with distilled water and stained overnight with 1% Alcian blue in 0.1 *N* hydrochloric acid (Paulsen and Solursh 1988). Cultures were washed twice rapidly in 0.1 *N* hydrochloric acid and stored in 70% ethanol at 4° C.

Quantitation of chondrogenesis was carried out by excising the culture chamber insert membrane with the stained explant attached, mounting it on a glass slide and reading the density of the Alcian blue stain using a Vickers M85 Scanning Microdensitometer with a spot size of 4 μm and a scan of 200 μm. Four readings were taken for each cartilage nodule in the culture, and the mean of these readings was taken as the value for each culture.

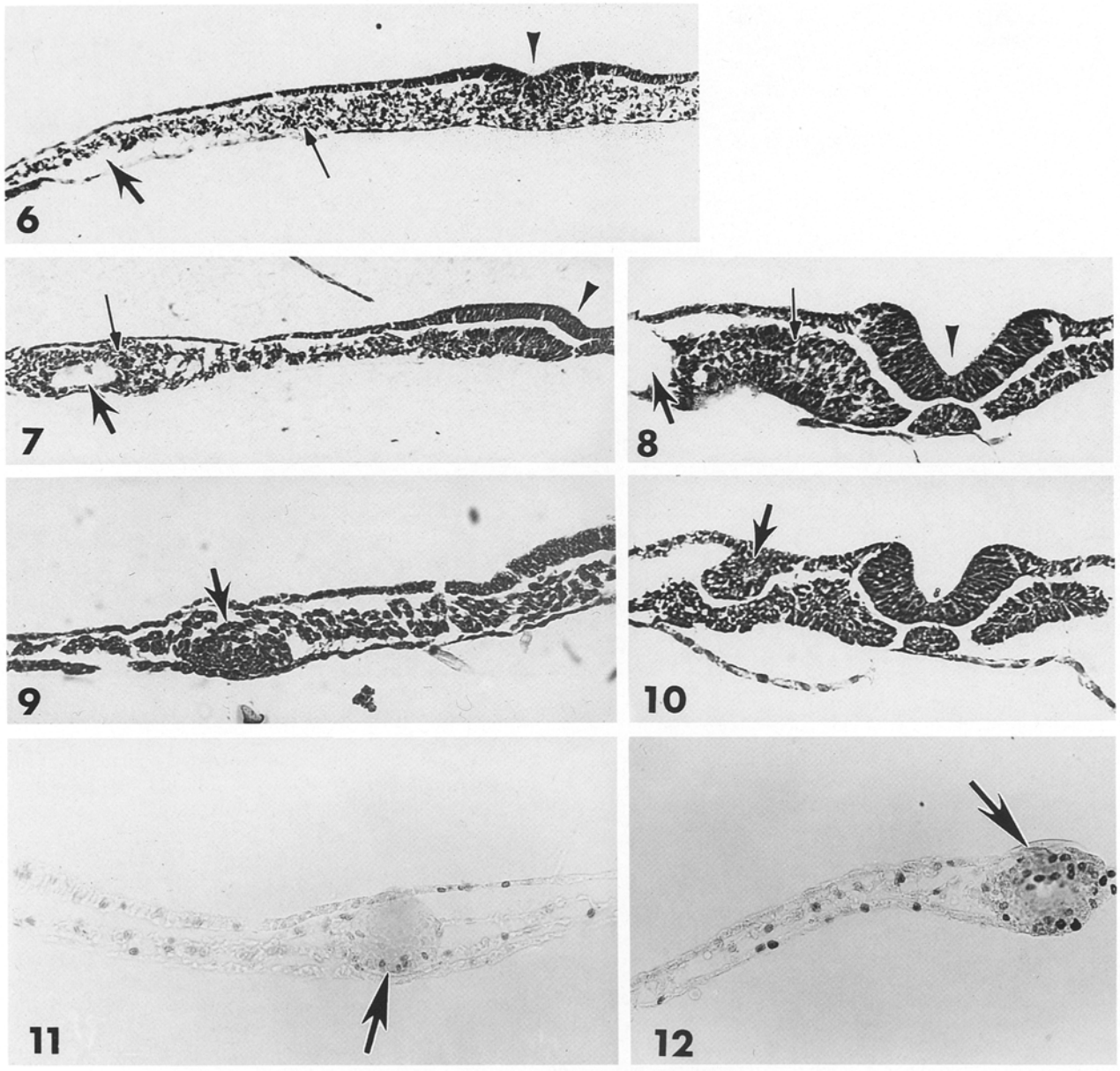


Fig. 6. Transverse section through a primitive streak stage embryo (stage 5) that had been implanted with an ion-exchange resin bead previously soaked in saline. The bead had been in place for 6 h prior to fixation, and was located just out of the plane of section at the position indicated by the *large arrow*. Because the process of sectioning frequently displaced the beads, sections used for illustration are taken from levels immediately adjacent to the position of the bead. The morphology of the primitive streak (*arrowhead*) and the disposition of the mesoderm cells (*small arrow*) are normal. × 100

Fig. 7. As Fig. 6, but the embryo was implanted for 6 h with an ion-exchange resin bead previously soaked in TGFβ1. The position occupied by the bead is indicated by the *large arrow*. Mesoderm cells (*small arrow*) have accumulated at the site of the implant. This embryo is at a slightly later stage (stage 6) than that in Fig. 5, having formed a neural plate (*arrowhead*). × 100

Fig. 8. As Fig. 7, but the bead was implanted close to the embryonic axis (*large arrow*). The embryo has proceeded to stage 7, and has formed an enlarged somite-like structure on the treated side (*small arrow*) in comparison with the contralateral side. Serial sections showed that the asymmetry was not due to an oblique plane of section. This embryo has a well formed and symmetrical neural groove (*arrowhead*). × 100

Fig. 9. This embryo was implanted with a piece of hydrolysed polyacrylamide previously soaked in TGFβ1. The site of the implant (*large arrow*), just out of the plane of the section, is characterized by an accumulation of mesoderm cells. × 100

Fig. 10. As Fig. 8, showing an overgrowth of ectoderm (*arrow*) overlying the implant site, which displaces the mesoderm. × 100

Fig. 11. Section through an embryo containing an untreated bead implant. After implantation, the embryo was exposed to BrdU for a 2-h period. Immunocytochemical localization of labelled nuclei shows that some cells, in the vicinity of the implanted bead and elsewhere, have incorporated the label (*arrow*). × 100

Fig. 12. As Fig. 11, except that the implanted bead (*arrow*) was pre-treated with TGFβ1. The cells around the bead have incorporated BrdU to a much greater extent than cells in surrounding areas. × 100

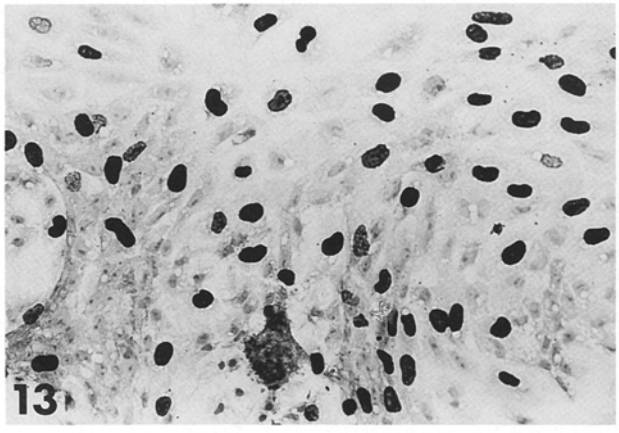
Results

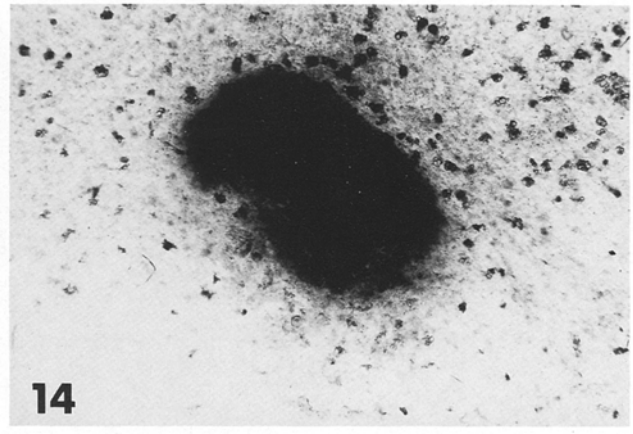
Effecs of anti-TGFβ1 on sclerotome cell spreading and extracellular matrix deposition in vitro

In a previous study (Sanders and Prasad 1991) it was shown that TGFβ1 could influence phenotypic transformation and extracellular matrix deposition by mesoderm cells in culture. Here, monoclonal antibody 1D11.16 was added to cultures of mesoderm or sclerotome cells grown on a fibronectin substratum, and produced a decrease in cell outgrowth, both in terms of a dose-dependent decrease in the overall size of resulting explants (Fig. 1), and as the degree of individual cell spreading (Figs. 2 and 3). The decrease in culture size was evaluated by counting the cell diameters surrounding the original explant mass after 1 day in culture. Controls, in which an irrelevant mouse monoclonal antibody was substituted for 1D11.16, were no different from controls containing no antibody at all, and all treatments were fully reversible by replacement of the antibody with normal medium. Assessment of fibronectin deposition by the antibody-treated cultures indicates that the decreased spreading was accompanied by decreased fibronectin deposition (Figs. 4, 5), while control cultures were unaffected.

Effects of TGFβ1-treated grafted beads in vivo

Previous work has shown that direct injection of TGFβ1 and 1D11.16 into embryos at the primitive streak stage does not give reproducible results (Sanders 1992). To introduce a localized application of TGFβ1 and its neutralizing antibody into the embryo, therefore, it was necessary to use the technique of grafting beads that had previously been soaked in these agents. In comparison with embryos that had been grafted with untreated beads (Fig. 6), embryos grafted with TGFβ1-treated beads showed an accumulation of mesoderm cells at the site of the implant (Fig. 7). This anomaly occurred in 37 out of 60 grafted embryos examined at gastrulation stages of development. In those embryos that had proceeded to neural plate stages, and where the grafted bead was placed close to the axis of the embryo, the effect on the mesoderm cells was such as to create a very large somite-like structure on the treated side (Fig. 8). This anomaly occurred in 18 out of 25 embryos at this stage of development. The effects on the mesoderm were produced by treated AG1-X2 ion-exchange beads in both the chloride and the formate forms. Pieces of hydrolysed polyacrylamide (Hypa, Zackson and Steinberg 1989), also soaked in TGFβ1, produced very similar effects on the mesoderm (Fig. 9). In no case did grafting of these





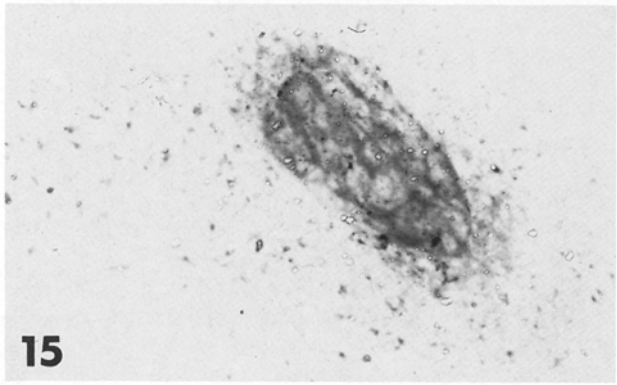


Fig. 13. Cultured explant of mesoderm cells from a gastrulating embryo. These cells were explanted in normal medium and exposed to 10 μg/ml BrdU for 2 h before fixing. Immunocytochemical localization of the incorporated BrdU is readily apparent, while unlabelled nuclei are visible owing to the very light background staining. Experimental cultures were exposed to TGFβ1 in the culture medium during BrdU incorporation. ×95

Fig. 14. Alcian blue-stained sclerotome/notochord co-culture after 6 days in the presence of medium containing 50 ng/ml TGFβ. A large mass of stained material is present, the density of which was determined by scanning microdensitometry. Small foci of Alcian blue staining are seen around the central mass. ×95

Fig. 15. As Fig. 14, except that the medium did not contain TGFβ. The density of the notochordal mass is much less than in medium containing TGFβ, and is quantified in Fig. 17. ×95

TGFβ1-treated materials result in the disruption of the embryonic axis or in the production of a secondary axis. In addition to the effects on the mesoderm cells, the only other anomaly found (11 out of 85 embryos) was an occasional overgrowth of the ectoderm overlying the graft (Fig. 10).

Effects of TGFβ1 on cell proliferation in vivo and in vitro

In order to determine if the aggregation of mesoderm cells in response to a TGFβ-treated bead implant involved cell proliferation in the immediate vicinity of the graft, gastrulating embryos containing a treated bead were incubated in the presence of BrdU. Immunocytochemical detection of incorporated BrdU indicated that cells in the locality of the implanted bead clearly showed a higher level of cell proliferation than those elsewhere in this region of the embryo (Fig. 12). Cells incorporating the label, although primarily in the mesoderm layer, were also present in the epiblast. Controls, implanted with beads not exposed to TGFβ1, showed no such elevation of incorporation (Fig. 11), indicating that the response was not due to wound healing.

In view of this result with beads grafted into the embryo in vivo, further experiments were carried out in vitro to examine the influence of TGFβ on the proliferation of mesoderm cells from the gastrulating embryo and of cells dissected from the sclerotome. Labelled nuclei were readily observable (Fig. 13). Rates of proliferation (Fig. 16), calculated as the percentage of labelled nuclei in a minimum of 1000 counted nuclei, were the same regardless of whether fibronectin, laminin or uncoated glass was used as a substratum. Although the sclerotome cells naturally proliferated at a faster rate than the gastrulation mesoderm, TGFβ1 had the same effect on both populations, causing a marked decrease in incorporation. Nuclei were scored for incorporation only in fully spread cells; cells within the unspread central mass of untreated explant tissue showed a much higher rate of incorporation than the spread cells, but it was not possible to measure accurately any response by this region of the explant. The effect of the antibody was equivocal, since it appeared to have little or no influence on the mesoderm, while somewhat reducing incorporation by the sclerotome.

Micromass cultures of sclerotome with notochord

Under the conditions used, each culture produced several nodules of Alcian blue-stainable cartilage after six days (Fig. 14). Control cultures (Fig. 15), lacking TGFβ1 in the medium, showed a much lower density of Alcian blue staining than cultures in which TGFβ1 was present (Fig. 14). The density of stain, determined densitometrically, was used as a measure of the level of chondrogenesis that had occurred in the presence of TGFβ1 and the 1D11.16 antibody (Fig. 17). In the pres-

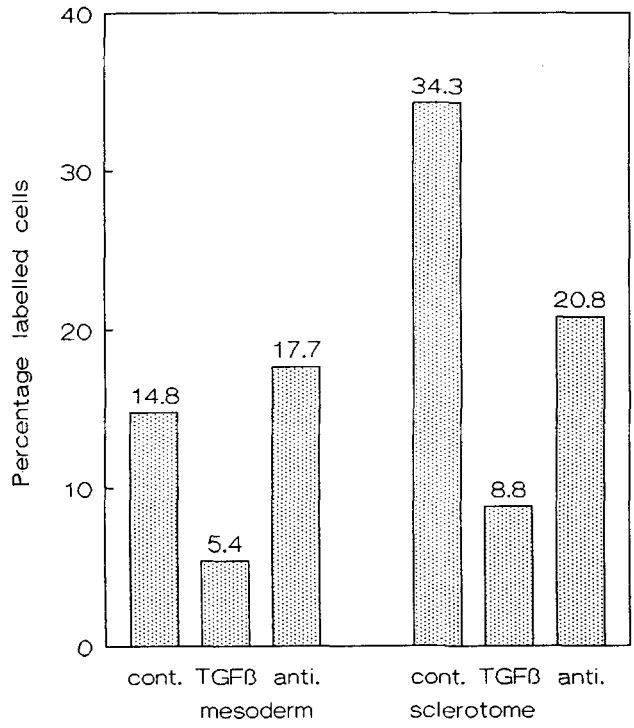


Fig. 16. The effect of TGFβ1 (50 ng/ml) and 1D11.16 (0.2 mg/ml) antibody on cell proliferation in vitro. Controls were cultured in normal medium. Proliferation of both cell types is inhibited by the presence of TGFβ1

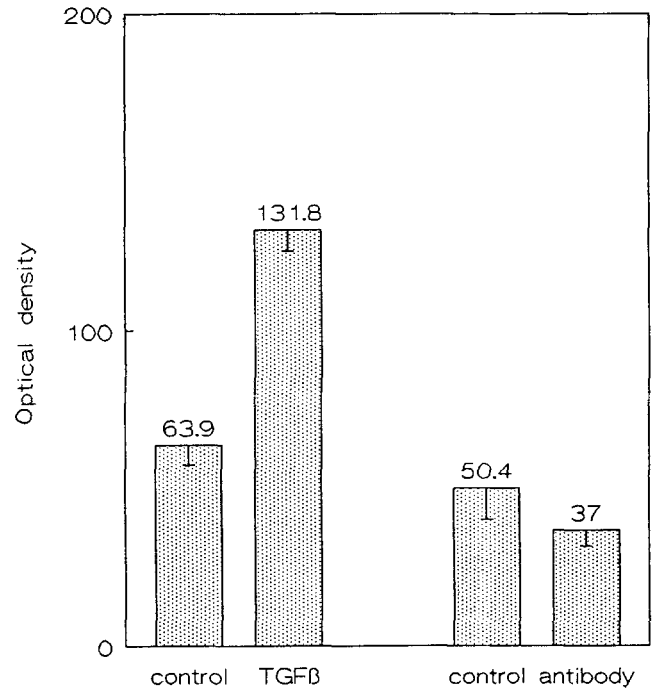


Fig. 17. The effect of TGFβ1 (50 ng/ml) and 1D11.16 (0.2 mg/ml) antibody on chondrogenesis in sclerotome/notochord co-cultures, estimated by measuring the optical density of the Alcian blue stain. Chondrogenesis is stimulated by the presence of TGFβ1

ence of TGFβ1 the mean density was 131.8 (±6.8 SE, $n=14$), compared with control values of 63.9 (±6.4 SE, $n=12$). This represents an increase of 106% under the experimental conditions. By contrast, in the presence of 1D11.16 antibody the values were 37.0 (±2.3 SE, $n=8$), compared to control mouse IgG values of 50.4 (±4.4 SE, $n=7$). This represents a decrease of 27% in the presence of the antibody.

Discussion

Previous results have shown that exogenously added TGFβ1 is able to effect a decreased adhesion of cultured chick embryo mesodermal cells to a fibronectin substratum, while reducing the deposition of fibronectin by the cells themselves (Sanders and Prasad 1991). By contrast, mesoderm cell adhesion to laminin was not affected, and fibronectin deposition by these cells on laminin was increased. The present results indicated that treatment of sclerotome cells on fibronectin with the TGFβ-neutralizing antibody 1D11.16 also reduced adhesion and fibronectin deposition. Thus, both raising and lowering the TGFβ levels in vitro appeared to have similar effects on these parameters. Parallels for this apparently paradoxical situation may be found in the bimodal effects of TGFβ on cell proliferation in a variety of other cell types where seemingly contradictory effects of TGFβ may be due to subtle differences in cell sub-population, cell shape, substratum or proliferative state (Moses et al. 1987; Sutton et al. 1991; Tang et al. 1991). The possibility has been considered previously (Moses et al. 1990) that such apparently conflicting evidence may also be a result of a concentration-dependence, as appears to be the case here. Regardless of the explanations of this effect, the current results confirm our previous observation that modulation of TGFβ levels in the embryo can influence both cell adhesion and extracellular matrix deposition by early embryonic mesoderm cells, and support the view that this factor is a significant regulator of early morphogenesis in the chick.

There is now ample precedent for the successful use of implanted ion-exchange resin or agarose beads as vehicles for the local delivery of agents to embryonic tissues (Tickle et al. 1985; Stern et al. 1990; Wedden et al. 1990; Hayamizu et al. 1991; Osmond et al. 1991). By means of this technique, we have clearly shown that the local application of TGFβ1 results in an aggregation of mesoderm cells in the vicinity of the graft and, in somite-stage embryos, results in the formation of oversized somites. That this is due, at least in part, to a stimulation of cell proliferation among the mesoderm cells is shown by the simultaneous increase in incorporation of BrdU by these cells. The validity of the result is indicated not only by the absence of such a result in the saline-treated controls, but also by the observation that implanted grafts of hydrolysed polyacrylamide (Zackson and Steinberg 1989) have the same effect. Since we have previously shown that TGFβ1 is present among the mesoderm cells (Sanders and Prasad 1991), it seems likely that stimulation of cell proliferation is one function of this factor in this location, and that this effect is integrated with other functions that we ascribed to it previously, such as modulation of cell adhesiveness and extracellular matrix deposition, to provide a comprehensive regulation of mesoderm morphogenesis (see also Choy et al. 1990). Although we cannot rule out the possibility that the mesoderm cell aggregation is also due in part to a chemotactic effect of TGFβ (Lucas and Caplan 1988; Eskandarani and Ayad 1989; Yang and Moses 1990), stimulation of cell proliferation is a general and primary effect of TGFβ in a variety of cell systems (Barnard et al. 1990; Massagué 1990; Moses et al. 1990) and is therefore not unexpected. On the other hand, in a comparable study using implanted slow-release plastic pellets containing TGFβ, Silberstein and Daniel (1987) demonstrated an in vivo inhibition of cell proliferation in mammary gland tissue, providing another example of discrepant results with this factor.

In contrast to the in vivo result, the in vitro study clearly indicated a growth-inhibitory effect for TGFβ1 on both gastrulation mesoderm and on sclerotome. Although there are many reports of growth inhibition by TGFβ1 (Yang and Moses 1990; Migdalska et al. 1991; Sutton et al. 1991; Tang et al. 1991), its paradoxical effects on cell proliferation are well documented (Barnard et al. 1990; Massagué 1990; Moses et al. 1990). The closest parallels to the present in vitro results are the data of Godin and Wylie (1991) and Rogers et al. (1992), who showed inhibition of proliferation by TGFβ1 of cultured primordial germ cells from the early mouse embryo, and of avian neural crest cells respectively. While Godin and Wylie (1991) proposed a concomitant chemotropic effect in vitro, we were only able to make such a suggestion from our in vivo results (see above). The explanation of the discrepant in vivo and in vitro data in the present case may well lie in the possibility that TGFβ normally acts in concert with other soluble factors which may modulate its effects in vivo. Further, it is possible that for the occurrence of a stimulatory response it is necessary for the tissue to retain a three-dimensional architecture, rather than the tissue culture monolayer morphology. Evidence for this may perhaps be seen in the observation that proliferation rate appeared to be higher in the unspread explant mass in the centre of each culture.

It has been reported that certain soluble factors are able to induce mesoderm formation and axis development experimentally in the chick in a similar fashion to the well-established experimental situation in amphibians (Mitrani et al. 1990). However, Mitrani and Shimoni (1990) showed that neither TGFβ1 nor TGFβ2 were among the factors that could, alone, induce the appearance of these structures from isolated epiblasts. On the other hand, TGFβ2 has been claimed to possess inductive properties in the chick embryo (Cooke and Wong 1991), although this evidence is not strong. In agreement with the results of Mitrani and Shimoni (1990), we have never observed supernumerary axis for-

mation, or any other effects on axial pattern formation, in response to the presence of TGFβ1, either directly applied or applied via carrier beads.

We have shown previously that TGFβ1 is detectable immunocytochemically in the sclerotome of 3-day embryos (Sanders 1992), as it is also in certain other chondrogenic tissues of the chick embryo (Jakowlew et al. 1991; Thorp et al. 1992). Our in vitro assays for the effect of TGFβ1 on the chondrogenic potential of the sclerotome in micromass culture indicated that there could be a stimulatory effect of this factor, which acts in concert with the inductive stimulus provided by the perinotochordal matrix. Such a conclusion is consistent with some previous evidence using different tissues, both in vitro (Kulyk et al. 1989; Hayamizu et al. 1991) and in vivo (Joyce et al. 1990). However, this is by no means a uniform finding (as discussed by Hayamizu et al. 1991), since other laboratories have reported either inhibition of chondrogenesis by TGFβ1 (Seyedin et al. 1988), or variable results depending upon a number of determinants, including the time-course of treatment and state of cell differentiation (Carrington and Reddi 1990; Leonard et al. 1991; Galéra et al. 1992). Most of these previous studies, however, have used chondrogenic tissue from embryonic limb buds; none have used sclerotome-notochord co-cultures as described here, and there is no reason to suggest that the two systems should be consistent with one another. As Hayamizu et al. (1991) point out, discrepancies in results between the different limb bud studies can most likely be accounted for by the different culture conditions. What we have shown is that TGFβ1 is both appropriately located, and is able, to influence chondrogenesis by the chondrocytes derived from the sclerotome. It is most unlikely that TGFβ acts alone in this activity, but how its effects are modulated by the presence of other factors in the embryo remains to be determined.

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