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Induction of chondrogenesis: requirement for synergistic interaction of basic fibroblast growth factor and transforming growth factor-beta

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SUMMARY

Interactions between the epithelial anlage of the developing mouse inner ear and its associated periotic mesenchyme control the differentiation of the cartilaginous otic capsule. Transforming growth factor-beta$_1$ (TGF-β_1) is a naturally occurring signal peptide that is present in these tissues at times of active differentiation and morphogenesis. Previous studies have shown that TGF-β_1 alone is not a sufficient stimulus to initiate chondrogenesis in cultured periotic mesenchyme. In this study, we provide evidence that basic fibroblast growth factor (bFGF) can elicit a specific but limited chondrogenic response in cultured periotic mesenchymal cells. We also demonstrate that simultaneous addition of bFGF and TGF-β_1 to cultured periotic mesenchyme results in a full chondrogenic response comparable to that which occurs when periotic mesenchyme is grown in the presence of its natural inductor tissue (i.e. otic epithelium). Utilizing antibodies directed against bFGF, we show localization of endogenous bFGF in the otic epithelium in vivo and in mixed epithelial-mesenchymal cultures. Additionally, we demonstrate the presence of FGF-like activity in medium conditioned by otic epithelium. Blocking of epithelial elicited chondrogenesis by a combination of both αbFGF and αTGF-β_1 antibodies provides further evidence of the necessity for these growth factors in the chondrogenic differentiation of periotic mesenchyme in vitro. Our results suggest a role for both bFGF and TGF-β_1 in the regulation of chondrogenesis during otic capsule formation in situ.

Key words: chondrogenesis, epithelial-mesenchymal interactions, bFGF, TGF-β_1.

INTRODUCTION

Interactions between embryonic tissues regulate the progression of specific cell populations through their differentiation pathways. During these inductive events, locally produced signal molecules can influence the developmental fate of targeted cell populations. Not only are these signal molecules present within the early embryo in vivo (Rapolee et al., 1988; Wilkinson et al., 1989; Slager et al., 1991), their effects on tissue interactions in situ may be mimicked in vitro by culture with exogenous growth factors (Sharpe and Ferguson, 1988). A growth factor that appears to have a significant involvement in early morphogenetic events is basic fibroblast growth factor (bFGF), a potent modulator of proliferation and differentiation in a wide variety of cell types (Gospodarowicz et al., 1987). Expression patterns of bFGF and FGF-related peptides during mouse embryogenesis demonstrate the participation of this multigene family in the control of cartilage growth and differentiation in situ (Kato, 1992). Moreover, exogenous bFGF is mitogenic for chondrocytes, demonstrating a specific effect on their differentiation in vitro (Gospodarowicz et al., 1987; Globus et al., 1988).

Deposition of the TGF-β family of signal peptides and expression of their respective mRNAs at sites of active differentiation and morphogenesis (Heine et al., 1987; Lehnert and Akhurst, 1988; Fitzpatrick et al., 1990; Pelton et al., 1990a,b) imply an active role for the members of the TGF-β family of growth factors in embryogenesis, particularly during epithelial-mesenchymal interactions. Morphogenesis of the mammalian inner ear is an example of a developmental process where epithelial-mesenchymal tissue interactions control the differentiation of the cartilaginous otic capsule (Frenz and Van De Water, 1991). TGF-β_1 peptide is present in both the epithelial and mesenchymal tissues of the developing inner ear at sites of incipient cartilage formation (Frenz et al., 1992). Moreover, TGF-β_1 has been shown to modulate the expression of chondrogenic phenotype by cultured periotic mesenchyme. However, TGF-β_1 alone has not been shown to function as an initiator of otic chondrogenesis (Frenz et al. 1992). Because bFGF and TGF-β_1 can coordinately regulate cellular functions such as proliferation and extracellular matrix accumulation (Gospodarowicz et al., 1987; Schweigerer et al., 1987), and molecules closely related to bFGF and TGF-β have been suggested to be the natural inducers of mesodermal tissue in vertebrate development (Kimelman and Kirschner, 1987; Weeks and Melton, 1987), we sought to determine whether otic

chondrogenesis can be effectively induced by the coordinated action of TGF-β_1 and bFGF. We were also interested in determining whether bFGF alone is a sufficient signal to initiate this inductive event.

We have investigated the chondrogenic response of high density cultures of periotic mesenchyme, isolated from embryonic age 10.5 day (E10.5) mouse embryos, to bFGF and examined whether TGF-β_1 can act synergistically with bFGF to potentiate its effects. Using antibodies directed against bFGF, we determined that endogenous bFGF is present in otic epithelium both in vivo and in vitro. Additionally, we tested for and confirmed the presence of FGF-like activity in medium conditioned by otic epithelium. Finally, by blocking the effects of endogenous bFGF and/or TGF-β_1 with antibodies directed against these growth factors, we demonstrated an inhibition of the chondrogenic response by cultured mesenchymal cells to otic epithelium. We provide evidence that there is a coordinated action of both endogenous bFGF and TGF-β_1, and that this synergistic interaction is necessary for the initiation of a full chondrogenic response in vitro. Thus, our results support the hypothesis that these endogenous growth factors may be natural regulators of epithelial-induced chondrogenesis during otic capsule formation in situ.

MATERIALS AND METHODS

Experimental animals

Hybrid CBA/C57 BL6 mouse embryos, obtained by crossmating CBA-J and C57 BL6-J mice (Jackson Laboratories), or CD-1 mouse embryos (Charles River) of gestation age E10.5, were utilized. Gestational age was estimated by the vaginal plug method, with the day of plug occurrence designated as day 1 (E1). After death of the gravid females by cervical dislocation, embryos were excised and immediately placed into Dulbecco's phosphate-buffered saline (PBS, Gibco). Embryonic age (E) was determined by somite count (i.e. E10.5=29-36 somites; Theiler, 1972).

Tissue culture

Periotic mesenchymal cells were prepared from the developing inner ears of El0.5 mouse embryos, resuspended in Ham's F-12 medium supplemented with l0% fetal bovine serum, and cultured at a density of 2.5×10^5 cells per l0 μl spot in 4 well tissue culture plates (Nunc l34673), as described by Frenz and Van De Water (1991). Basic FGF (bovine, recombinant, Boehringer Mannheim) or a combination of bFGF and human platelet derived TGF-β_1 (Assoian et al., 1983) was added into culture 1 hour after cell seeding for a period of 48 hours. The concentration of added bFGF, or bFGF in combination with TGF-β_1, was varied between 0.5 ng to 10 μg/ml, but was routinely used at 1 ng/ml. To determine the specificity of the effects of basic FGF, epidermal growth factor (EGF; 1-5 ng/ml; mouse submaxillary gland derived, Boehringer Mannheim), platelet-derived growth factor (bPDGF; 1-5 ng/ml; human recombinant, United States Biochemical Corporation) or TGF-β_1 (1-5 ng/ml) was also introduced into cultured mesenchyme 1 hour after cell seeding for a 48 hour period. Additionally, bFGF, TGF-β_1, or PDGF were added to cultures at molar concentrations of 2×10^{-3} M, 1×10^{-4} M, 2×10^{-4} M, or 5×10^{-5} M. In control cultures, growth factors were not administered. Medium was changed every 48 hours. Cultures were maintained for a total incubation period of 7 days. In mesenchymal cultures containing otic epithelium, periotic mesenchyme and otic epithelium were dissected, dissociated, and cultured as described (Frenz and Van De Water, 1991). Antibodies directed against TGF-β_1 peptides (Flanders et al., 1988), αbFGF neutralizing antibodies (Kurokawa et al., 1989), or a combination of these antibodies were added to the cultures 1 hour after cell seeding and maintained in the cultures for the duration of the culture period (i.e. 7 days). The concentration of αTGF-β_1 was varied between 1-25 μg/ml, but was routinely used at 10 μg/ml. The concentration of αbFGF typically introduced into a culture was 4 μg/ml. Combinations of αTGF-β_1 and αbFGF antibodies standardly comprised 4 μg/ml αbFGF and 10 μg/ml αTGF-β_1. In antibody neutralization studies, bFGF (1 μg/ml) was preincubated with αbFGF antibodies (4 μg/ml; 30 minutes at 37°C), and introduced on the initial day of culture to E10.5 periotic mesenchyme.

Antibody preparation

Rabbit polyclonal antibodies to unconjugated peptides corresponding to the amino-terminal 30 amino acids of TGF-β_1 were prepared as described (Flanders et al., 1988). In enzyme-linked immunosorbent assays, western blots, and immunoprecipitation assays, antisera demonstrated no reactivity against TGF-β_2 (Flanders et al., 1988). Neutralizing (sheep or rabbit polyclonal) antibodies to recombinant bFGF (Kurokawa et al., 1989) were generously provided by Drs Patricia Farber and Michael Klagsbrun (Children's Hospital, Harvard University, Boston, MA). SDS-PAGE analysis demonstrated that the αbFGF antibody is highly specific, cross-reacting with bFGF, but not acidic FGF (Kurokawa et al., 1989).

Quantitation of mesenchymal condensations

Cultures were monitored at low magnification on an Olympus CK2 or Nikon Diaphot inverted microscope for identification of cell condensations using phase contrast and Hoffman modulation contrast microscopy (Frenz et al., 1992; Frenz et al., 1989). Between culture days 3 and 4, condensations were counted. These counts were compared with mean condensation counts in mesenchymal cultures containing otic epithelium (Frenz and Van De Water, 1991).

Quantitative Alcian blue staining of cultures

Cultures were fixed after 7 days in vitro with a solution of 10% formalin containing 0.5% cetylpyridinium chloride (CPC) and then stained with Alcian blue 8GX at pH 1.0, as described (Frenz et al., 1992; Frenz and Van De Water, 1991). After cell spots were washed with a 3% solution of acetic acid (adjusted to pH 1.0 with HCl), bound stain, an index of accumulated sulfated glycosaminoglycans (S-GAG; Lev and Spicer, 1964), was then extracted with an 8 M guanidine hydrochloride solution and measured by spectrophotometric quantitation (Hassell and Horigan, 1982) using a Cambridge Technology Inc. microplate reader equipped with a 600 nm wavelength filter. Comparison of S-GAG accumulation in bFGF-treated and bFGF+TGF-β_1-treated cultures was made to mesenchymal cultures that were grown in the presence of otic epithelium, as described (Frenz and Van De Water, 1991).

Incorporation of radiolabeled sulfate into S-GAG

Mesenchymal cells were grown as described, but with the addition of 2 μCi of $Na^{35}SO_4$ per ml (carrier free; 1 Ci=37 GBq: Amersham) to the culture medium. Medium was collected at every change and pooled for each cell spot. After a period of 6 days in vitro, each cell spot was homogenized in 1 ml of medium. Homogenates and pooled media were digested overnight (37°C) with 100 μg of proteinase K per ml. Fifty microliter aliquots of each digest were spotted onto strips of Whatman 3 MM filter paper as described (Frenz et al., 1992; Leonard et al. 1991). After drying completely, these strips were washed 5 times in 1% CPC in 0.3 M NaC1 (Wasteson et al., 1973). Strips were dried, cut, then suspended in Aquasol and counted 3 times on a Beckman liquid scintillation counter.

Determination of DNA content in mesenchymal cultures

A modification of the method of LaBarca and Paigen (1980) was used for DNA determination. Cell spots were harvested on culture day 7 and samples homogenized in phosphate-saline buffer (0.05 M $NaPO_4$,

2.0 M NaC1, 2×10^{-3} M EDTA, pH 7.4). Aliquots of the homogenate (20 μg) were mixed with 2 ml phosphate-saline buffer containing 10 μl of compound Hoechst 33258. Fluorescence measurements were made on a Hoefer Scientific Instruments DNA fluorometer (TKO 100). The assay was checked for linearity by making dilutions of calf thymus DNA (i.e. a reference standard; ICH Laboratories, Inc..) and plotting a standard curve. Optical density readings correspond to DNA concentrations in the μg/ml range.

In vitro radiolabeling of cells and determination of relative cell proliferation

Cultures were prepared as described and grown in the presence of 1.0 μCi of [^{3}H]thymidine per ml (Amersham TRK.686) in medium with or without added bFGF (1 ng/ml). Radioactive medium was replaced every 48 hours. After 7 days in culture, acid-precipitable radioactivity in each cell layer was determined by liquid scintillation counting.

Detection of type II collagen in culture

After a period of 7 days in vitro, collagen was extracted from cultures of E10.5 mesenchyme, or E10.5 mesenchyme containing otic epithelium, by constant stirring in 0.5 ml of 0.5 M acetic acid at 4°C overnight. Detection of type II collagen in the extract was determined by indirect antibody enzyme-linked immunosorbent assay (ELISA) using an ELISAmate kit (Kirkegaard and Perry Laboratories Inc.). Briefly, 100 μl aliquots of the collagen extract (serially diluted in 0.5 M acetic acid) were added to the wells of a 96-well plate (Nunc-Immuno plate, Maxisorp) and allowed to air dry at room temperature. In positive control wells, known reference samples of type II collagen (bovine; Southern Biotechnology Associates, Inc.) in 0.5 M acetic acid were utilized. Following addition to each well of a blocking solution containing 1% bovine serum albumin in phosphate-buffered saline (5 minutes), 100 μl of affinity purified goat α-type II collagen antibody (Southern Biotechnology) was applied to the wells and allowed to react for 1-2 hours at room temperature. In negative control wells, sample or primary antibody was omitted. Wells were washed 3 times each with a solution containing 0.02% Tween, and 100 μl horseradish peroxidase (HRP)-labeled rabbit anti-goat IgG (Southern Biotechnology) added to each well for 1 hour. After washing the wells 3 times each, 100 μl peroxidase substrate solution was dispensed into each well and allowed to react for 30 minutes at 37°C. Plates were read on a Biotechnology microplate reader equipped with a 405 nm wavelength filter. The color produced (i.e. blue-green reaction product) corresponded to the amount of type II collagen in the sample.

Immunohistochemical staining in situ

The distribution of bFGF was determined by immunohistochemical staining utilizing the avidin-biotin complex method (Vector kit). Cross sections were prepared from E10.5 mouse inner ears as described below. Specimens were fixed in methacarn fixative (Puchtler et al., 1970), dehydrated in methanol, cleared in methyl benzoate (Fisher) and Histoclear (National Diagnostics), and embedded in paraffin (Surgipath) at 59°C. Specimens 5-6 μm thick were collected on alcohol-cleaned, uncoated slides and deparaffinized. Nonspecific binding was blocked by preincubation with normal rabbit serum (30 minutes, 20°C). Sections were incubated with αbFGF antibodies diluted 1:150 in Tris-buffered saline (pH 7.6) at 4°C overnight in a humidified chamber. Controls were done by replacing the αbFGF sheep IgG with bovine serum albumin. Following a final incubation with 3,3′-diaminobenzidine, some specimens were counterstained with Mayer's hematoxylin (Sigma). Specimens were mounted in crystal mount (Biomedia) with final mounting in Permount (Fisher).

Indirect immunofluorescence in vitro

Mesenchymal cultures containing otic epithelium were grown on glass coverslips that were inverted into the wells of 4-well tissue culture plates. Cultures were fixed in cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 minutes and then washed three times for 5 minutes each with 0.1 M phosphate buffer. The antibody directed against recombinant bFGF was applied for 30 minutes at room temperature. Cells were washed free of primary antibody with 0.1 M phosphate buffer. Controls were prepared by omitting application of primary antibodies to the samples or by replacing antibodies with either preimmune serum or bovine serum albumin. A secondary antibody conjugated to fluorescein isothiocyanate (FITC) was then applied for 30 minutes at room temperature. Cells were washed free of secondary antibody, and the coverslips mounted in glycerol/phosphate buffer (9:1). Specimens were examined for the presence of bound antibody on a Zeiss Axiophot using a FITC epi-fluorescence blue wavelength (450-490 nm) excitation filter set.

Preparation of conditioned medium

One hundred and seventy otocysts were dissected from El0.5 mouse embryos as previously described (Frenz and Van De Water, 1991) and cultured at 37°C in 1 ml Ham's F-12 medium for 41 hours. Epithelial tissue was removed from the medium, and the medium was assayed for the presence of FGF-like activity (described below).

Isolation of FGF-like factors from conditioned medium

One ml of conditioned F-12 medium was diluted to 3 ml with 0.1 M Hepes buffer, pH 7.2. The sample was applied to a heparin-Sepharose column previously equilibrated with the same buffer, as previously described (Gordon et al., 1989). The column was rewashed with 20-30 ml of the 0.1 M Hepes buffer, then with buffer containing 0.5 M NaC1 to remove TGF-β and PDGF. FGF-like growth factors were eluted with Hepes buffer containing 2.0 M NaC1 and collected in 1 ml fractions.

Endothelial cell proliferation

Endothelial cells from human umbilical vein were plated in 12-well Falcon tissue culture plates at a density of 4×10^4 cells/well in medium 199 containing 14 moles of partially purified bFGF and 25 μg/ml of heparin. On day 2 the medium was removed, and a 300 μl aliquot of the fraction containing FGF-like growth factors eluted from the heparin-Sepharose column (described above) was added to the medium 199 that contained 25 μg/ml of heparin but not FGF. Positive control wells received medium 199 supplemented with 14 pmoles of partially purified bFGF and 25 μg/ml heparin. Cells in negative control wells were grown either in unconditioned F-12 medium alone or in medium 199 supplemented with 25 μg/ml of heparin. Cells were harvested with trypsin after 4 days in culture and counted in a Coulter counter (Gordon et al., 1984). All assays were performed in triplicate.

RESULTS

Mesenchymal condensation formation in response to basic FGF

We have previously shown that precartilage mesenchymal condensations, similar in appearance to those associated with otic chondrogenesis in situ, develop in cultures of E10.5 periotic mesenchyme containing otic epithelium, but not in cultures of E10.5 mesenchyme alone (Frenz and Van De Water, 1991). We introduced exogenous bFGF (i.e. in place of otic epithelium) into high-density cultures of E10.5 mesenchyme. Addition of bFGF at a concentration of 1 ng/ml (48 hour exposure) resulted in the development of a few mesenchymal condensations (Fig. 1A,B). Mean values for the number of condensations, given in Table 1, was 86% less than that which occurred in the presence of otic epithelium (Table 1; also see Frenz and Van De Water, 1991). Lower concentrations of bFGF (i.e. 0.5 ng/ml) failed to

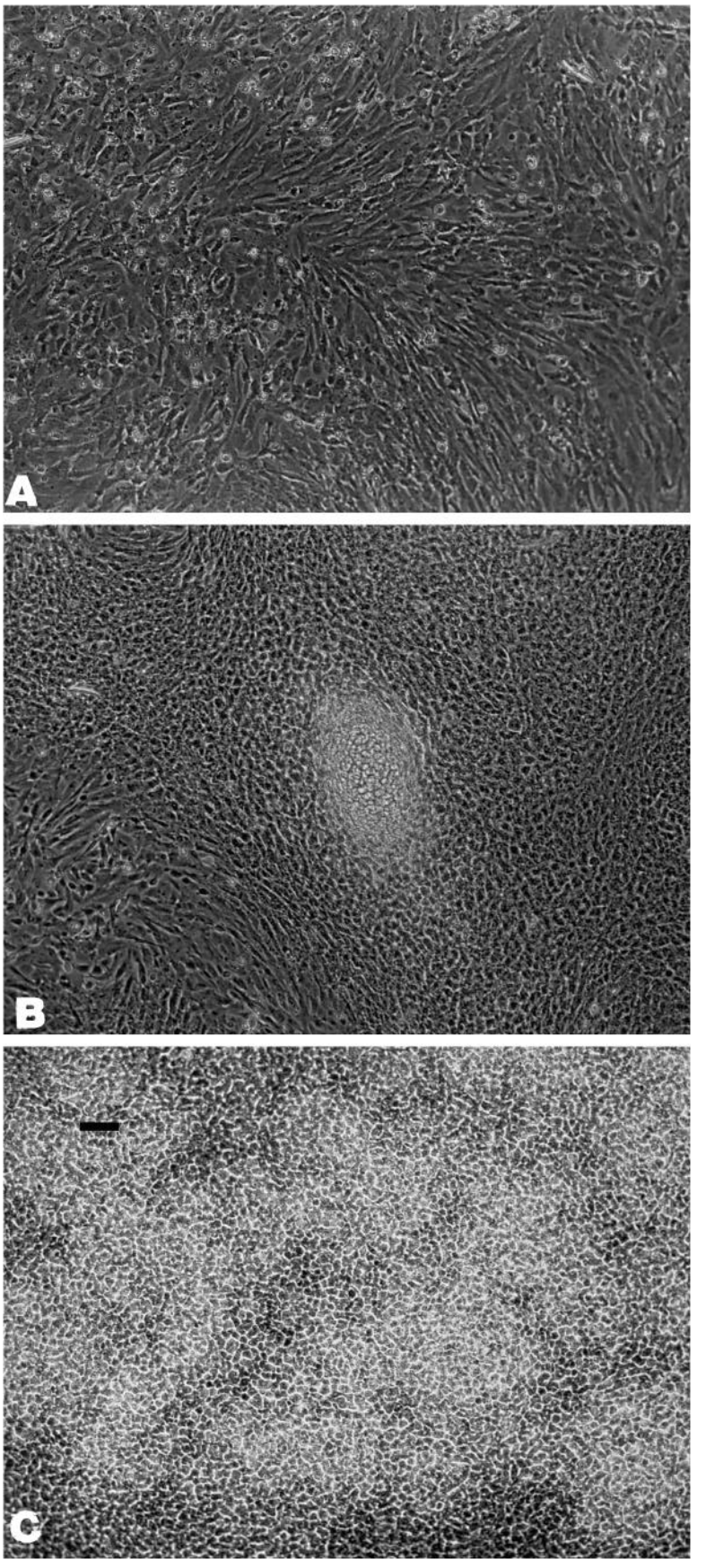

Fig. 1. High-density cultures of E10.5 periotic mesenchyme (7 days in vitro), (A) without added growth factors (i.e. bFGF or TGF-β_1), showing the absence of a chondrogenic response by the mesenchymal cells; (B) grown in the presence of bFGF (1 ng/ml, 48 hours); (C) grown in the presence of bFGF+TGF-β_1 (1 ng/ml each, 48 hours) Comparison of the isolated chondrogenic foci in B with the multiple, coalescing foci in C demonstrates a marked enhancement of chondrogenesis in response to the simultaneous addition of bFGF and TGF-β_1. Phase contrast micrographs. Bar, 35 μm.

initiate condensation formation, while higher concentrations (i.e. between 2.5-10 ng/ml) resulted in condensation numbers comparable to those observed at 1 ng/ml. A representative experiment is shown in Fig. 2. In addition, exposure of cultured mesenchyme to bFGF for durations greater than 48 hours (i.e. 72-96 hours) did not result in an increase or decrease in condensation number in comparison to cultures exposed to bFGF for a 48 hour period. However, exposure to bFGF (1 ng/ml) for only 24 hours was not sufficient to initiate the cellular condensation process. The effect of bFGF on the formation of cellular condensations appeared to be specific, since addition of 1 ng/ml of either TGF-β_1 (see also Frenz et al. 1992), PDGF, or EGF did not result in their development (Table 1). Similarly, higher concentrations of TGF-β_1, PDGF, or EGF (i.e. 2 ng/ml or 5 ng/ml) did not initiate condensation formation. Furthermore, because PDGF and TGF-β_1 are approximately twice the relative molecular mass of bFGF, the effects of these growth factors were also compared based on a molar concentration. When bFGF, PDGF, or TGF-β_1 were added to E10.5 mesenchymal cultures at concentrations of 2×10^{-3} M, 1×10^{-4} M, 2×10^{-4} M, or 5×10^{-5} M, cellular condensations developed only in the presence of bFGF, and were comparable in numbers to those observed at 1 ng/ml. Moreover, when E10.5 mesenchymal cultures were exposed to bFGF (1 ng/ml), which was pretreated with αbFGF neutralizing antibodies (4 μg/ml), mesenchymal condensations did not form even after 7 days in vitro.

Basic FGF does not affect proliferation of cultured periotic mesenchyme

Rather than directly affecting cell differentiation, bFGF may have initiated the condensation process in cultured mesenchyme by influencing the rate of cell proliferation, thereby changing the cell density of these cultures. To address this possibility, we measured DNA content and acid-insoluble incorporation of [^{3}H]thymidine in cultures grown in the presence or absence of this growth factor (1 ng/ml). Comparison of values for bFGF-treated cultures with non-treated cultures indicated there were similar quantities of DNA (μg/ml) under both culture conditions (Table 2). In accord with these findings, there were no significant differences between bFGF-treated and non-treated cultures in incorporation of labeled precursor (Table 2).

Table 1. Effects of growth factors on cellular condensation in cultured E10.5 mesenchyme

EGF*	PDGF	TGF-β_1	bFGF	Otic epithelium†	bFGF + EGF	bFGF + PDGF	bFGF + TGF-β_1
0	0	0	4±3	28±3	4±3	5±2	27±4

Values represent the mean numbers of condensations counted between day 3 and 4 in 3-6 cultures per experimental group.
*****Growth factors were added at a concentration of 1 ng/ml.
†In all mesenchymal cultures containing otic epithelium, equivalent amounts of epithelial tissue were present.

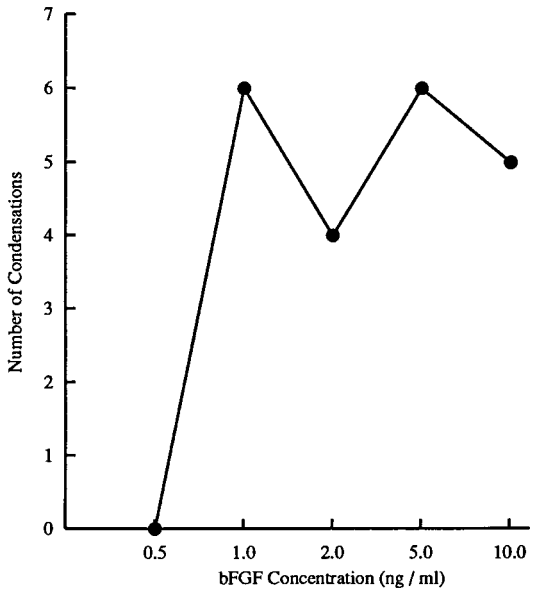

Fig. 2. A representative dose response of cultured E10.5 periotic mesenchyme to exogenous bFGF. Mesenchymal condensations did not form at 0.5 ng/ml, while a similar number of condensations developed at concentrations between 1-10 ng/ml.

Effects of a combination of bFGF and TGF-β_1 on condensation formation in cultured periotic mesenchyme

Because the number of mesenchymal condensations that develop in the presence of bFGF are markedly less than that which occurs in the presence of inner ear epithelium, factors other than bFGF may also be involved in the control of otic chondrogenesis. To test this possibility, we added growth factors in combination with bFGF to high-density cultures of El0.5 periotic mesenchyme. Neither EGF (1 ng/ml, 48 hours) nor PDGF (1 ng/ml, 48 hours) in combination with bFGF enhanced the chondrogenic response in the cultured mesenchyme (Table 1). In contrast to these results, TGF-β_1 (1 ng/ml) did potentiate bFGF-induced chondrogenesis (Table 1; Fig. lC), increasing the number of cell condensations formed by 75% compared to cultures treated only with bFGF. Addition of TGF-β_1 at concentrations greater than 1 ng/ml, in combination with bFGF at 1 ng/ml, did not further stimulate the condensation process. Moreover, only simultaneous addition of TGF-β_1 and bFGF resulted in an enhanced chondrogenic response by the mesenchymal cells. Introduction of bFGF into culture either prior to or following the addition of TGF-β_1 did not result in an increase in the mesenchymal condensation number. Furthermore, the time of addition of these growth factors was critical. To stimulate chondrogenesis, TGF-β_1 and bFGF were required for a 48 hour period beginning on the initial day of culture. Addition of these factors at a time following the day of cell seeding (i.e. days 1-3) did not stimulate the condensation process.

Table 2. Effects of bFGF on cellular proliferation in cultured E10.5 mesenchyme

Mesenchyme	bFGF
A. DNA content (μg/ml)*	
19±1.4	19±0.8
B. [^{3}H] thymidine incorporation (cpm)	
14,067±2281	14,022±2366

All values represent the mean for 2-3 cultures per experimental group.
*****Cultures of mesenchyme alone (i.e control) and cultures of mesenchyme treated with bFGF (1 ng/ml) were (A) assayed for DNA content by microfluorometric quantitation, with absorbance values expressed as DNA concentration in μg/ml, or (B) grown in culture medium containing 1 μCi of [^{3}H]thymidine per ml, with acid-precipitable radioactivity determined by liquid scintillation counting.

Effects of bFGF or a combination of bFGF and TGF-β_1 on S-GAG accumulation in periotic mesenchymal cultures

If bFGF and TGF-β_1 are natural regulators of epithelial-induced chondrogenesis, we reasoned that their effects on the condensation process (described above) should be reflected in the later chondrogenic event of S-GAG accumulation (Frenz et al., 1992; Frenz and Van De Water, 1991). Therefore, we examined the extent of chondrogenesis in response to bFGF, or a combination of bFGF and TGF-β_1, by measuring the accumulation of Alcian blue-positive matrix at pH 1.0. As indicated in Table 3, binding of Alcian blue stain in these growth factor-treated cultures was quantitated and compared with results for mesenchyme cultures grown in the presence and absence of otic epithelium. Cultures treated with bFGF alone bound Alcian blue stain, although to a limited extent due to the few chondrogenic sites that developed in these cultures (Fig. 1B; Table 1). This binding, while representing a 60% increase in S-GAG accumulation in comparison to non-treated mesenchyme cultures (Table 3), was only 23% of that which occurred in the mesenchyme cultures with otic epithelium

Table 3. Effects of bFGF and bFGF + TGF-β_1 on S-GAG accumulation

Mesenchyme	bFGF*	bFGF + TGF-β_1	otic epithelium
0.020±0.0007	0.050±0.02	0.251±0.047	0.216±0.019

Values represent the mean optical densities of matrix bound Alcian blue stain following extraction with 8 M guanidine-HC1 for 3 cultures per experimental group.
*****Basic FGF (1 ng/ml) or a combination of bFGF (1 ng/ml) + TGF-β_1 (1 ng/ml) were added to cultures of mesenchyme alone.

Table 4. Incorporation of $^{35}SO_4^{-2}$ into glycosaminoglycans in mesenchyme cultures

Treatment*	Fraction	cpm**
Control	cell layer	46±21
	medium	199±94
bFGF	cell layer	141±61
	medium	174±65
bFGF + TGF-β_1	cell layer	266±75
	medium	202±25
Otic epithelium	cell layer	303±101
	medium	216±16

*****Control cultures (i.e. mesenchyme alone), cultures of mesenchyme containing otic epithelium, and cultures of mesenchyme treated with bFGF (1 ng/ml) or a combination of bFGF (1 ng/ml) + TGF-β_1 (1 ng/ml), were maintained in culture medium containing 2 μCi of $Na^{35}SO_4$/ml for a period of 6 days in vitro.

******Each value represents the mean for 2-3 cultures per experimental group.

(Table 3; see also Frenz and Van De Water, 1991). However, when a combination of bFGF and TGF-β_1 was introduced into cultured E10.5 mesenchyme, binding of Alcian blue stain, and therefore S-GAG accumulation, was comparable in extent to that which occurred in the presence of otic epithelium (Table 3; see also Frenz and Van De Water, 1991). Previous studies have shown that the continuous presence of TGF-β_1 alone also stimulates E10.5 periotic mesenchyme to accumulate a limited amount of Alcian blue positive matrix (Frenz et al. 1992).

The observed increase in S-GAG accumulation by a combination of bFGF and TGF-β_1 may represent an effect of these growth factors on matrix retention rather than an increase in matrix production. To address this possibility, we labeled S-GAG using $^{35}SO_4^{-2}$ in non-treated (i.e. mesenchyme) cultures, or in mesenchyme cultures treated with a combination of bFGF and TGF-β_1, or bFGF alone. All radioactivity incorporated into CPC-precipitable GAG of the cell spots at 6 days in culture (Wasteson et al., 1973; Leonard et al., 1991; Frenz et al., 1992) and of the pooled medium collected over the 6 day culture period was separately assayed. Representative experiments are given in Table 4. The relative amounts of S-GAG in bFGF-treated, bFGF+TGF-β_1-treated, and non-treated mesenchyme cultures (i.e. cell layers) corresponded to the Alcian blue staining results in Table 3. The amounts of S-GAG exported into the medium of the bFGF-treated, bFGF+TGF-β_1-treated, and non-treated mesenchyme cultures were comparable, indicating that bFGF or the combination of bFGF and TGF-β_1 did not act by altering matrix retention.

In situ localization of bFGF

An antibody raised against recombinant bFGF defined the localization of this growth factor in the epithelial tissues of the E10.5 inner ear (Fig. 3). Reaction product is concentrated over the otocyst epithelium and neuroepithelium of the adjacent rhombencephalon. Only a diffuse reaction product is present over the cells of the periotic mesenchyme.

Immunolocalization of basic FGF in mesenchymal cultures containing otic epithelium

We have previously shown that endogenous TGF-β_1 is present in cultured epithelium of the E10.5, E12, and E14 mouse inner ear (Frenz et al., 1992). The correspondence between initiation

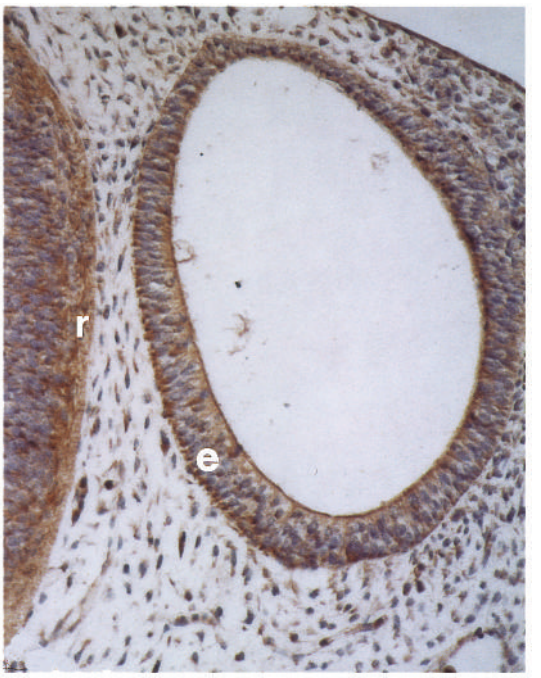

Fig. 3. E10.5 inner ear. αbFGF antibodywas used to show immunoreaction product concentrated over the epithelium (e) of the developing otocyst and the neuroepithelium of the neighboring rhombencephalon (r).

of chondrogenesis by otic epithelium and by bFGF now led us to investigate the localization of endogenous bFGF in mesenchyme cultures containing otic epithelium. On culture day 1, E10.5 mesenchymal cultures containing otic epithelium were fixed and immunofluorescently labeled for bFGF. Prior to examining the cultures for the presence of bound αbFGF, cultures were examined by differential interference contrast microscopy to identify mesenchyme and epithelium (Fig. 4A). The presence of staining for bFGF was detected only over the otic epithelial tissue and not in the surrounding mesenchyme (Fig. 4B). When similar day-2 to -3 cultures were immunofluorescently labeled with αbFGF, bFGF was detected in the surrounding mesenchyme as well (Fig. 4C). In control specimens, no immunostaining of either tissue occurred.

Effects of endogenous basic FGF and/or TGF-β_1 are blocked by antibodies

To determine the necessity of endogenous bFGF and/or TGF-β_1 for the initiation of chondrogenesis by cultured E10.5 mesenchyme, αTGF-β_1 (10 μg/ml) and/or αbFGF (4 μg/ml) antibodies were added to cultures of E10.5 mesenchyme containing otic epithelium (i.e. inductor tissue), and maintained for the duration of the culture period (i.e. 7 days). Between culture days 3 and 4, mesenchymal condensations were counted and compared to non-treated mesenchyme cultures containing otic epithelium (Table 5). When a combination of αbFGF (4 μg/ml) and αTGF-β_1 (10 μg/ml) was introduced into E10.5 mesenchyme cultures containing otic epithelium, the formation of mesenchymal condensations was totally inhibited (Table 5). Consequently, chondrogenesis, as measured by S-GAG accumulation, did not occur (Table 6). In contrast,

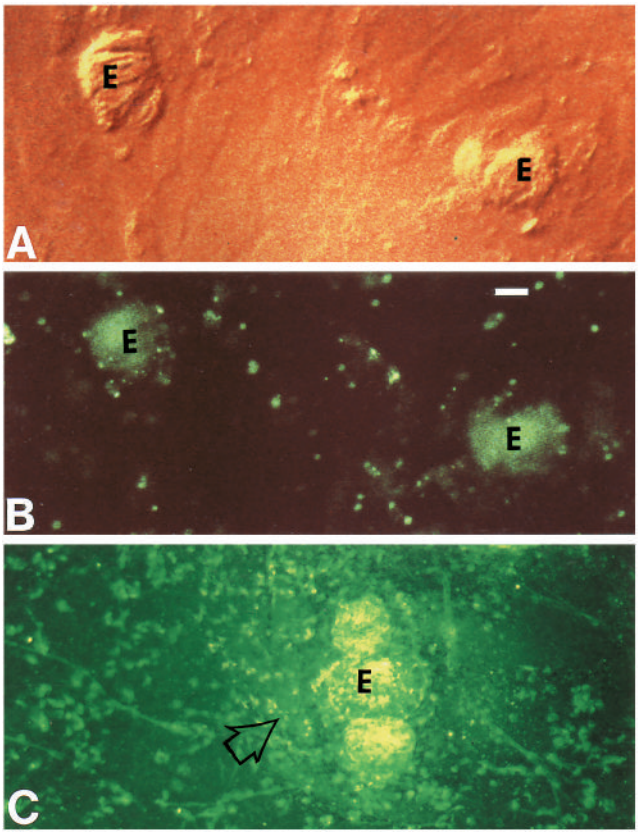

Fig. 4. Immunolocalization of endogenous bFGF in vitro. (A) A differential interference contrast micrograph of a day 1 culture of E10.5 periotic mesenchyme containing otic epithelium (E). (B) An immunofluorescent micrograph of the same field, showing localization of endogenous bFGF in the otic epithelium (E) only. (C) An immunofluorescent micrograph of a similar day 2 culture of E10.5 periotic mesenchyme containing otic epithelium now shows the binding of αbFGF antibody to both the otic epithelium and the surrounding periotic mesenchyme (arrow). Bar, 35 μm.

Table 5. Effects of α-TGF-B$_1$ and αbFGF antibodies on mesenchymal cell condensations in cultures of El0.5 mesenchyme + epithelium

Control	αEGF*	αTGF-β_1	αbFGF	αTGF-β_1 + αbFGF
37±12	38±4	21±11	3±2	0

Values represent mean numbers of condensations counted between day 3 and 4 in 3-9 cultures per experimental group.
*αEGF or αTGF-β_1 were introduced at a concentration of l0 μg/ml; αbFGF at 4 μg/ml.

addition of either αbFGF (4 μg/ml) or αTGF-β_1 (10 μg/ml) to the mesenchyme with epithelium cultures resulted in some suppression, but not a total inhibition, of the condensation process. A comparison of experimental values with those of non-treated controls indicated that condensation number was decreased by 92% and 43% in the presence of αbFGF or αTGF-β_1 respectively (Table 5), but not in the presence of αEGF antibodies, where the number of condensations formed were comparable to control culture levels (Table 5). Correspondingly, after a period of 7 days in vitro, comparison of non-treated cultures

Table 6. Effects of αbFGF and/or αTGF-β_1 antibodies on S-GAG accumulation in cultures of El0.5 mesenchyme + epithelium

Control	αTGF-β_1*	αbFGF	αFGF + αTGF-β_1
0.212±0.04	0.082±0.04	0.022±0.004	0.008±0.004

Values represent the mean optical densities of matrix bound Alcian blue stain for 5-6 cultures per experimental group.
*αTGF-β_1 was added at a concentration of l0 μg/ml; αbFGF at 4 μg/ml.

with cultures grown in the presence of αbFGF or αTGF-β_1 antibodies indicated a 90% and 61% decrease in binding of Alcian blue stain (i.e. S-GAG accumulation; Table 6). Concentrations of αTGF-β_1 as high as 50 μg/ml or αbFGF at 40 μg/ml also resulted in decreased condensation numbers but did not completely suppress their formation. In addition, when αTGF-β_1 was introduced into cultures at a concentration as low as 5 μg/ml and αbFGF at a concentration of 0.4 μg/ml, condensation numbers were decreased but not as dramatically.

Effects of bFGF and/or TGF-β_1 are specific for chondrogenic differentiation

To determine the specificity of the effects of bFGF and/or TGF-β_1 on chondrogenic differentiation in cultured E10.5 periotic mesenchyme, the expression of type II collagen, a cartilage-specific macromolecule (Miller, 1984), was examined. Acetic acid extracts from cultures of mesenchyme alone or cultures of mesenchyme grown in the presence of either bFGF (1 ng/ml) or TGF-β_1 (1 ng/ml), or a combination of bFGF (1 ng/ml) and TGF-β_1 (1 ng/ml) were assayed by ELISA on culture day 7 to demonstrate and quantify the expression of type II collagen. When treated with bFGF, type II collagen was expressed by the cultured cells (Table 7), with the levels of this macromolecule increasing by 50% when cultures were exposed to a combination of bFGF and TGF-β_1 (Table 7). However, in cultures of mesenchyme alone or TGF-β_1-treated cultures of E10.5 periotic mesenchyme, levels of type II collagen were only 0.02% and 0.023% of that detected in the bFGF+TGF-β_1-treated mesenchyme (Table 7). Moreover, when a combination of αbFGF (4 μg/ml) and αTGF-β_1 (10 μg/ml) antibodies were introduced into E10.5 mesenchyme cultures containing otic epithelium, levels of type II collagen, as assayed by ELISA, were reduced by 98.4% when compared to non-treated cultures of mesenchyme and otic epithelium (Table 7). In the presence of either αTGF-β_1

Table 7. Expression of type II collagen in chondrogenic cultures

A. Treatment*	Quantity**	B. Treatment	Quantity
Control	0.06±0.007	Otic epithelium	3.7±0.08
TGF-β_1	0.07±0.01	αTGF-β_1	1.8±0.06
bFGF	1.5±0.28	αbFGF	1.3±0.07
bFGF + TGF-β_1	3.0±0.21	αbFGF+αTGF-β_1	0.06±0.01

*Acetic acid extracts of (A) cultures of mesenchyme that were grown in the presence or absence of bFGF (1 ng/ml) and/or TGF-β_1 (1 ng/ml) and (B) mesenchymal cultures containing otic epithelium that were grown in the presence or absence of αbFGF and/or αTGF-β_1 were assayed by ELISA for type II collagen.
**Quantity of type II collagen is expressed in μg/ml. Values represent the mean for 2-3 cultures per experimental group.

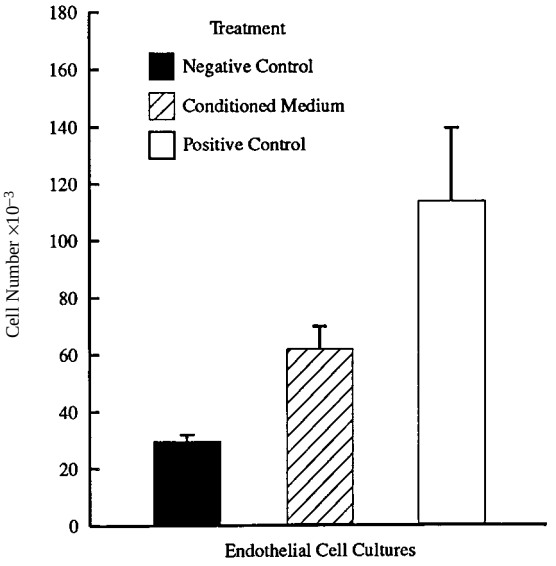

Fig. 5. Comparison of the effects of medium supplemented with 25 μg/ml of heparin (negative control); medium containing 14 pmoles of bFGF and 25 μg/ml of heparin (positive control), and medium conditioned with otic epithelium, on the mitogenic response by cultured human umbilical endothelial cells. Depicted values represent the mean cell count for 3 cultures per experimental group.

(10 μg/ml) or αbFGF (4 μg/ml) antibodies, levels of this macromolecule decreased 51% and 65% respectively compared to non-treated mesenchyme and otic epithelium cultures (Table 7).

Mitogenic activity by FGF-like factors in conditioned medium

Heparin-sepharose column chouromatography of medium conditioned with otic epithelium yielded an eluate that contained FGF-like material. When this eluate material was added to an endothelial cell culture test system, significant cell proliferation occurred. The mitogenic effect of the conditioned medium in these cultures was greater than twice the response seen with medium supplemented with heparin (i.e. negative control cultures; Fig. 5).

DISCUSSION

In previous work, we found that E10.5 periotic mesenchyme differentiated into cartilage when cultured in the presence of otic epithelium (Frenz and Van De Water, 1991). When TGF-β_1, an endogenous epithelial factor in the E10.5 inner ear, was substituted for this inductive tissue, the mesenchymal cells accumulated a limited Alcian-blue-positive ECM, but failed to form chondrogenic foci (Frenz et al., 1992). These observations support the hypothesis that TGF-β_1 alone cannot act as a sufficient signal to initiate otic capsule chondrogenesis. In this study, we examined the possibility that the maximal initiation of chondrogenic events during inner ear formation requires the synergistic interaction between TGF-β_1 and another growth factor.

We report here that bFGF alone can specifically initiate chondrogenesis in cultured E10.5 mesenchyme (Tables 1, 3 and 7). This chondrogenic response to bFGF, which can be blocked by the addition of neutralizing αbFGF antibodies, is substantially less effective than that evoked by otic epithelium, the natural inductor tissue (Tables 1, 3; see also Frenz and Van De Water, 1991). Because doses of bFGF ranging from 0.5 ng/ml to 10 μg/ml were tested, it is unlikely that this limited chondrogenic response is a function of growth factor concentration (Fig. 2). Rather, this discrepancy suggests that the signal(s) required to initiate an otic chondrogenic response by the cultured periotic mesenchyme can only be partially mimicked by the addition of bFGF.

When we examined the ability of bFGF to coordinately act with several other signal peptides, we found that simultaneous addition of TGF-β_1 with bFGF to culture medium potentiated the mesenchyme's response to bFGF's inductive effects, increasing both the number of mesenchymal condensations and, correspondingly, the extent of chondrogenesis (measured by Alcian blue staining at pH 1.0 and $^{35}SO_4^{-2}$ incorporation) to levels comparable to that induced by the natural inductor tissue, i.e. otic epithelium (Tables 1, 3, 4). The cellular response, in accord with the findings of Saksela et al. (1987), is dependent upon both the order and time of addition of the growth factors. In addition, $^{35}SO_4^{-2}$ incorporation into GAG (Table 4) indicated that the marked increase in S-GAG accumulation by the combination of bFGF and TGF-β_1 resulted from effects on S-GAG synthesis, not matrix retention. Moreover, the increase in type II collagen levels in response to these signal molecules (Table 7) demonstrated the specificity of their synergistic effects on chondrogenic differentiation. Previous studies have shown that rabbit articular chondrocytes produce large amounts of type II collagen following treatment with bFGF and TGF-β (Inoue et. al., 1989).

The synergistic interaction of bFGF with TGF-β_1 appears to be specific since the other growth factors tested (i.e. PDGF, EGF) could not even minimally stimulate bFGF-induced chondrogenesis (Table 1). This is in accord with the studies of Kato et al. (1987), where TGF-β potentiated the growth response of sternal chondrocytes to bFGF, but TGF-β in combination with EGF did not produce a synergistic effect. TGF-β increases the efficiency of FGF in stimulating chondrocyte proliferation in soft agar (Iwamoto et al., 1989), while these growth factors also act additively to enhance chondrogenic differentiation in high-density cultures of limb bud mesenchyme (Schofeld and Wolpert, 1990). Furthermore, regulation of chick limb outgrowth by a combination of FGF-4 and BMP-2, a member of the TGF-β superfamily (Niswander and Martin, 1993), and mesoderm induction by bFGF and TGF-β in isolated animal pole blastomeres, (Kimelman and Kirschner, 1987) also demonstrate a coordinate regulation of cellular functions by these families of growth factors. Hence, synergy of these factors may be a general principle in embryonic development. Additionally, exposure of limb mesenchyme to exogenous TGF-β_1 in vitro or in vivo results in a marked enhancement (Kulyk et al., 1989; Leonard et al., 1991) or ectopic expression (Hayamizu et al., 1991) of chondrogenesis respectively. However, opposing effects of bFGF and TGF-β_1 have also been reported (Baird and Durkin, 1986; Saksela et al., 1987; Stocker et al., 1991; Elford and Lamberts, 1991).

Int-2, an FGF-related proto-oncogene, is expressed in the

epithelial tissues of the E10.5 mouse otic vesicle (Wilkinson et al., 1989). Targeted disruption of int-2 results in transgenic mice with developmental defects of the inner ear that correlate with disruption of int-2 expression in the otic epithelium (Mansour et al., 1993). Basic FGF and TGF-β_1 are also present in the epithelial anlage of the developing inner ear both in vivo and in vitro (Figs 3, 4B,C; Frenz et al., 1992). When antibodies directed against either TGF-β_1 or bFGF, but not other growth factors (i.e. EGF), were introduced into El0.5 mesenchymal cultures containing otic epithelium, epithelial-induced chondrogenesis was markedly but not totally suppressed (Tables 5, 6, 7). The processes of cellular condensation and S-GAG accumulation could only be completely blocked by a combination of αTGF-β_1 and αbFGF antibodies (Tables 5, 6), emphasizing not only the synergy between bFGF and TGF-β_1 but the necessity for the participation of both these signal peptide factors in otic chondrogenesis. Moreover, a 98.4% decrease in type II collagen levels in the presence of αbFGF and αTGF-β_1 as compared to non-treated cultures demonstrates the specificity of their synergistic effects on chondrogenic differentiation (Table 7). Furthermore, in accord with the studies of Represa et al. (1991), where αint-2 antibody inhibited the formation of the otic vesicle from the otic placode, inhibition of chondrogenesis by αTGF-β_1 and αbFGF suggests that these growth factors must be secreted to elicit their inductive effects. However, while int-2, kFGF, and FGF-5 genes code for a signal sequence for secretion (Dixon et al., 1989; Acland et al., 1990; Delli-Bovi et al., 1988; Zhan et al., 1988), the bFGF gene is significantly different, and lacks a conventional signal peptide sequence, leaving to question how bFGF is directed extracellularly (Gospodarowicz et al., 1987; Folkman and Klagsbrun, 1987). Recent studies suggest that cellular secretion of bFGF could occur in association with ECM components such as heparin sulfate (Schweigerer et al., 1987). Results of our endothelial cell proliferation assay indicate the presence of secreted FGF-like factors in otic epithelial-conditioned medium (Fig. 5).

Since bFGF can exert mitogenic effects on cultured bone cells (Gospodarowicz et al., 1987; Globus et al., 1988), and TGF-β potentiates these mitogenic effects (Globus et al, 1988), we examined the possibility that the response of periotic mesenchyme to bFGF may involve similar proliferative effects. Evidence that initiation of otic chondrogenesis does not involve growth control is provided by the fact that bFGF does not change the overall quantity of DNA or the rate of incorporation of [^{3}H]thymidine in cultured mesenchyme (Table 2). This is in accord with previous studies, in which FGF did not alter the rate of incorporation of ^{3}[H]thymidine into the DNA of animal cap cells (Slack et al., 1987). Although induction of otic chondrogenesis by bFGF most probably occurs by direct cellular effects, the synergistic function of TGF-β_1 could be intimately linked with other mechanisms, such as regulating the composition of the extracellular matrix (Massague, 1987; Ignotz et al., 1986) and the ability of the mesenchymal cells to interact with it (Massague, 1987). Moreover, in potentiating the inductive effects of bFGF, TGF-β_1 may alter either the cellular distribution or binding affinity of bFGF receptors (Massague, 1987). An increase of bFGF receptors in response to TGF-β_1 has previously been implicated in positive modulation of cellular responses to bFGF by TGF-β_1 (Lefebvre et al., 1991; Baird and Bohlen, 1989). Alternatively, bFGF has been shown to enhance TGF-β_1 gene expression in osteoblast-like cells (Noda and Vogel, 1989).

In conclusion, we have demonstrated synergy between bFGF and TGF-β_1 in the initiation of chondrogenesis in high-density cultures of early periotic mesenchyme. Localization of bFGF and TGF-β_1 in otic epithelium (Frenz et al., 1992), and suppression of otic epithelial-initiated chondrogenesis by antibodies directed against these growth factors support a role for bFGF and TGF-β_1 as signal molecules that mediate capsule formation in situ. In future studies, we are interested in determining whether otic epithelial cells produce other members of the TGF-β and FGF families of growth factors, e.g. BMP-2a and FGF-3 (i.e. int-2) respectively, that may mediate epithelial-mesenchymal interactions in this system.

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