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Original Article

Effects of dentin proteins, transforming growth factor ß1 (TGFß1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast *in vitro*

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ABSTRACT We have studied the effects of dentin proteins, of Transforming Growth Factor beta 1 (TGFß1) and Bone Morphogenetic Protein (BMP2) on the differentiation of odontoblasts *in vitro*. The total EDTA-soluble fraction of dentin proteins, prepared from rabbit incisors was further separated by chromatography on DEAE-Cellulose and heparin-agarose columns. While the total EDTA-soluble fraction of dentin had no effect on cultured dental papillae, fractions retained on both columns were able to initiate functional differentiation of preodontoblasts of isolated day-17 first lower mouse molar dental papillae cultured *in vitro*. TGFß1 and BMP2, both stimulated the matrix secretion by dental papillae cells. TGFß1 and BMP2, combined with the inactive total EDTA-soluble fraction, stimulated odontoblast differentiation. An active fraction retained on DEAE-Cellulose completely lost the inductive activity after incubation with a neutralizing anti-TGFß antibody. These results demonstrate that a TGFß-like molecule present in dentin could interact with some component which acts as a modulator of its activity on the initiation of the cytological and functional differentiation of odontoblasts.

KEY WORDS: *odontoblast-differentiation, TGFβ, BMP, dentin proteins*

Introduction

Odontoblasts are aligned in a single layer at the periphery of the dental papilla, and when fully differentiated, are tall columnar post-mitotic cells with a polarized distribution of organelles. Functional odontoblasts synthesize and secrete extracellular matrix constituents of predentin and dentin (Butler *et al.*, 1981; Linde, 1989). The combination of specific morphological, cytological and functional features determines the identity of odontoblasts (for review see Ruch, 1985).

Terminal differentiation of odontoblasts occurs in a specific temporospatial pattern within each tooth: in the first lower molar of laboratory-raised Swiss mice, the first overtly differentiated odontoblasts are observed on day-18 of gestation (vaginal plug= day 0). These cells are located at the tip of the main cusps and functional odontoblasts progressively develop towards the basal part of the cusps. Post-mitotic cells become functional after a few hours and their terminal differentiation is characterized by the following steps: odontoblasts withdraw from the cell cycle, polarize and secrete predentin-dentin.

Experimental data suggested that odontoblast terminal differentiation could only be achieved after a minimum number of cell cycles (Ruch *et al.*, 1976, 1982). Furthermore it was demonstrated that the inner dental epithelium exerts control over the terminal differentiation of odontoblasts (Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1982) through basement membrane-mediated interactions (Meyer *et al.*, 1977; Osman and Ruch, 1981; Ruch *et al.*, 1983). The operative basement membrane might work as a specific substrate (Lesot *et al.*, 1981, 1985, 1992) and/or as a reservoir for paracrine factors (Ruoslahti and Yamaguchi, 1991; Cam *et al.*, 1992; Schubert, 1992).

Abbreviations used in this paper: BMP, bone morphogenetic protein; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetracetic acid; FITC, fluorescein isothiocyanate; µm, micrometer; mRNA, messenger ribonucleic acid; nm, nanometer; rh, recombinant human; rs, recombinant simian; TGFß, transforming growth factor beta; Tris, Tris (hydroxymethyl) aminoethane.

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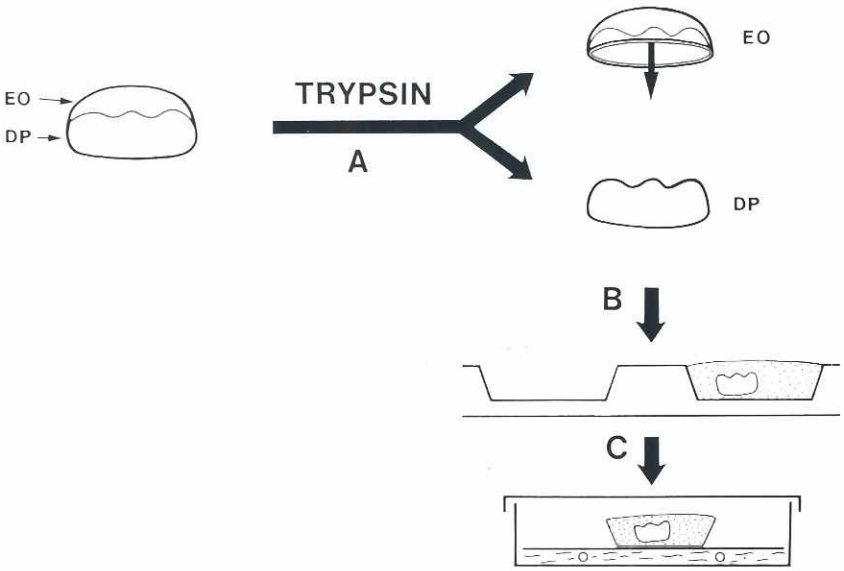


Fig. 1. The dental papilla *(DP) was mechanically separated from the enamel organ (EO) after trypsin dissociation of tooth germs* **(A)** *and embedded in 12 µl of semi-solid medium* **(B)**. *The dental papilla embedded in the semi-solid medium was then cultured for 6 days in Trowell-type cultures in 2.5 ml of liquid medium* **(C)**, *which was changed every 2 days.*

It is not known yet whether distinct odontoblast and pulp progenitor cell populations exist in the dental papilla. All the neural crest-derived dental papillae cells might be potential odontoblasts, although only some of them overtly differentiate according to a specific spatial distribution of epigenetic signals (Ruch, 1987).

Several authors have studied the biological properties of dentin matrix. It has been shown to contain proteins with mitogenic properties (Finkelman *et al.*, 1990) as well as chondrogenesis and osteogenesis stimulating activity (Harada *et al.*, 1990; Rabinowitz *et al.*, 1990). The biological effects of these proteins on dental mesenchymal cells have been investigated *in vivo* (Smith *et al.*, 1990; Tziafas and Kolokuris, 1990; Tziafas *et al.*, 1992) and *in vitro* (Lesot *et al.*, 1986). *In vivo* experiments have demonstrated that implantation of isolated dentin proteins (Smith *et al.*, 1990) and dentin matrix (Nakashima, 1990; Tziafas *et al.*, 1992) in dental pulp can directly induce odontoblast-like cell differentiation and synthesis of matrix which may indirectly further control cell polarization.

In vitro experiments, performed with isolated dental papillae cultured on Millipore filters coated with dentin proteins, showed the maintenance of polarized odontoblasts for several days, although these experimental conditions never afforded proof of the differentiation of preodontoblasts (Lesot *et al.*, 1986).

In this paper, we report the effects of dentin proteins on the morphological features of isolated dental papillae embedded in semi-solid medium containing agar and grown *in vitro*. Furthermore, the effects of the as yet uncharacterized dentin proteins were compared to those of recombinant human Bone Morphogenetic Protein 2: rhBMP2 (Wozney *et al.*, 1988), and of recombinant simian Transforming Growth Factor ß1: rsTGFß1 (Massagué, 1990; Sporn and Roberts, 1990). TGFß1 has been shown to be synthesized in developing teeth by means of both immunolocalization (Cam *et al.*, 1990) and *in situ* hybridization (Vaahtokari *et al.*, 1991); the latter technique also allowed detection of BMP2 mRNA (Lyons *et al.*, 1990). The effects of BMP2, formerly BMP2A, and TGFß1 have been extensively analyzed on bone cells (Centrella *et al.*, 1987; Joyce *et al.*, 1990; Yamaguchi *et al.*, 1991) and muscle cells (Harada *et al.*, 1990; Yamaguchi *et al.*, 1991), but no reports have been published concerning their possible action on dental tissues.

Results

The first lower molars of day-17 mouse embryos were dissociated and the dental mesenchymes (dental papillae) embedded in semi-solid medium (Fig. 1) prior to culture for up to 6 days. The morphological changes in the cells of these control cultures were compared to those of dental papillae embedded in semi-solid medium containing dentin proteins. The effects of the total EDTA-soluble fraction of dentin proteins were also compared to those of the same fraction of dentin proteins separated on either DEAE-Cellulose or heparin-agarose.

Dental papillae cultured in control media

Despite a progressive involution in the size of the dental papilla, cusp morphology could be maintained after 6 days in the semi-solid control medium (Fig. 2A,D,G,J,M). Furthermore, preodontoblasts localized at the periphery of the explant never became functional (Fig. 3G,H). Collagen type I (Fig. 4A) and fibronectin (Fig. 4B) were synthesized by dental mesenchymal cells but no preferential accumulation was observed at the periphery of the explants. Otherwise, the secretion of collagen type II was never detected in control conditions (not shown).

Dental papillae cultured in the presence of dentin proteins

When dental papillae were cultured in the presence of either total EDTA-soluble dentin proteins (Fig. 2B,E,H,K,N) or proteins retained on DEAE-Cellulose (Fig. 2C,F,I,L,O) cusp morphology was maintained. Significant involution was observed when dental papillae were cultured for 6 days in the presence of total EDTA-soluble fractions of dentin proteins (Fig. 2N) but not when cultured in the presence of proteins retained on DEAE-Cellulose (Fig. 2O). Immunofluorescent staining of dental papillae cultured in the presence of total EDTA-soluble proteins with antibodies directed

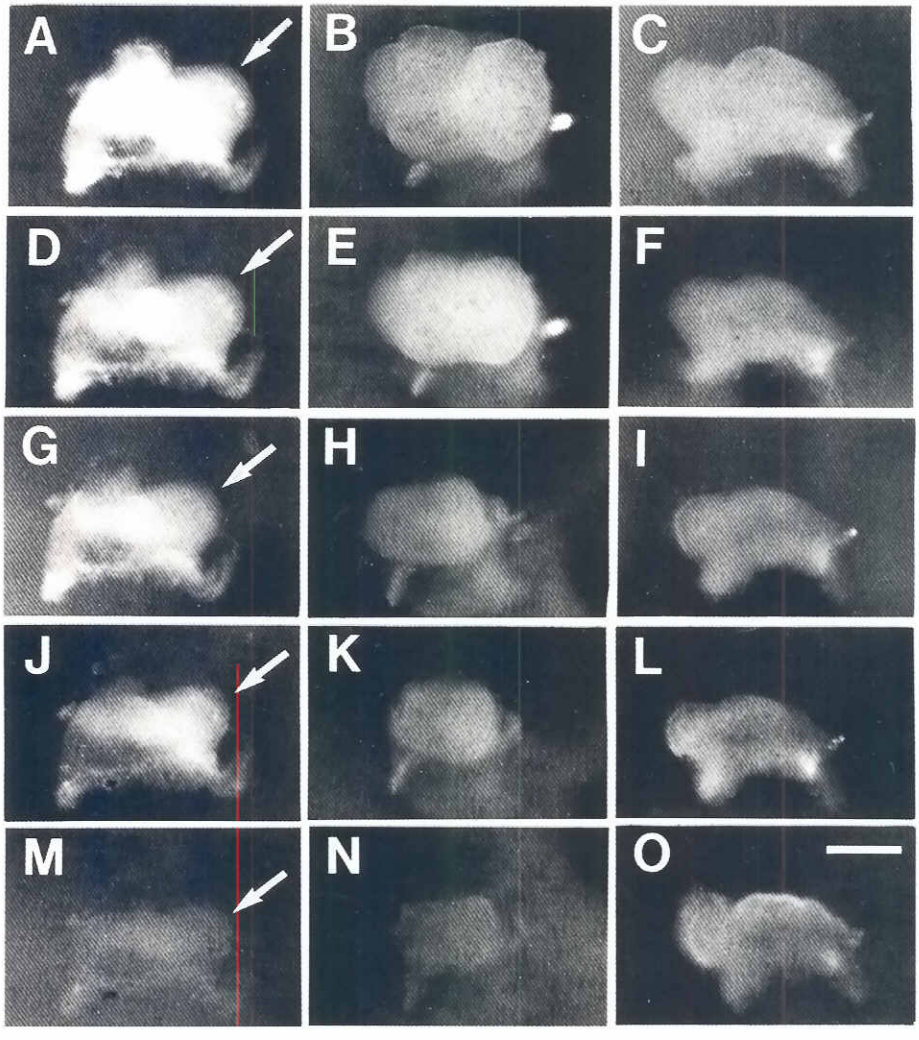


Fig. 2. Evolution of cuspal morphology in isolated dental papillae (A-C) dissociated from day-17 mouse first molars and cultured for one (D-F), three (G-I), four (J-L), and six days (M-O) observed with a dissecting microscope. *Cultures were performed in semi-solid culture medium containing either proteins of the total EDTA-soluble fraction of dentin (B, E, H, K, N), EDTA-soluble dentin proteins retained on DEAE-cellulose (C, F, I, L, O) or in the absence of dentin proteins (A, D, G, J, M). A progressive involution of the tissues was observed mainly in the presence of the total EDTA-soluble fraction of dentin proteins (B, E, H, K, N); however the cusp pattern was maintained after 6 days of culture in all cases (M, N, O). Arrows indicate the evolution of one cusp in one specimen. Scale bar= 200 μm.*

against either collagen type I (Fig. 4C), fibronectin (Fig. 4D) or collagen type II (not shown) did not show any differences when compared to control cultures (Fig. 4A,B).

DEAE-Cellulose

The total EDTA-soluble fraction of dentin proteins was loaded on a column of DEAE-Cellulose and sequentially eluted with a gradient of NaCl (Fig. 5A). The heterogeneous content of the different peaks has been demonstrated previously (Lesot *et al.*, 1986). The effects of these proteins eluted in the different peaks were compared to those of total EDTA-soluble dentin proteins. All the fractions (Table 1) allowed the initiation of odontoblast polarization after 4 to 6 days of culture (Fig. 3C,D). The polarized odontoblasts were functional: the cells deposited large amounts of extracellular matrix in contact with the semi-solid culture medium containing proteins (Fig. 3C,D). In most cases, when differentiation occurred, cusps pre-existing at the onset of the culture (Fig. 3A,B) were maintained and gradients of odontoblast differentiation were observed from the top to the bottom of the cusps (Fig. 3C). When dental papillae were cultured in the presence of total EDTA-soluble dentin proteins (Fig. 3E,F) polarized and functional odontoblasts were never observed (Table 1), and the volume of the cultured tissues decreased (Fig. 3E,F). The polarized cells accumulated collagen type I at the periphery of the explant when cultured in the presence of active fractions of dentin proteins (Fig. 4E), but no fibronectin (Fig. 4F).

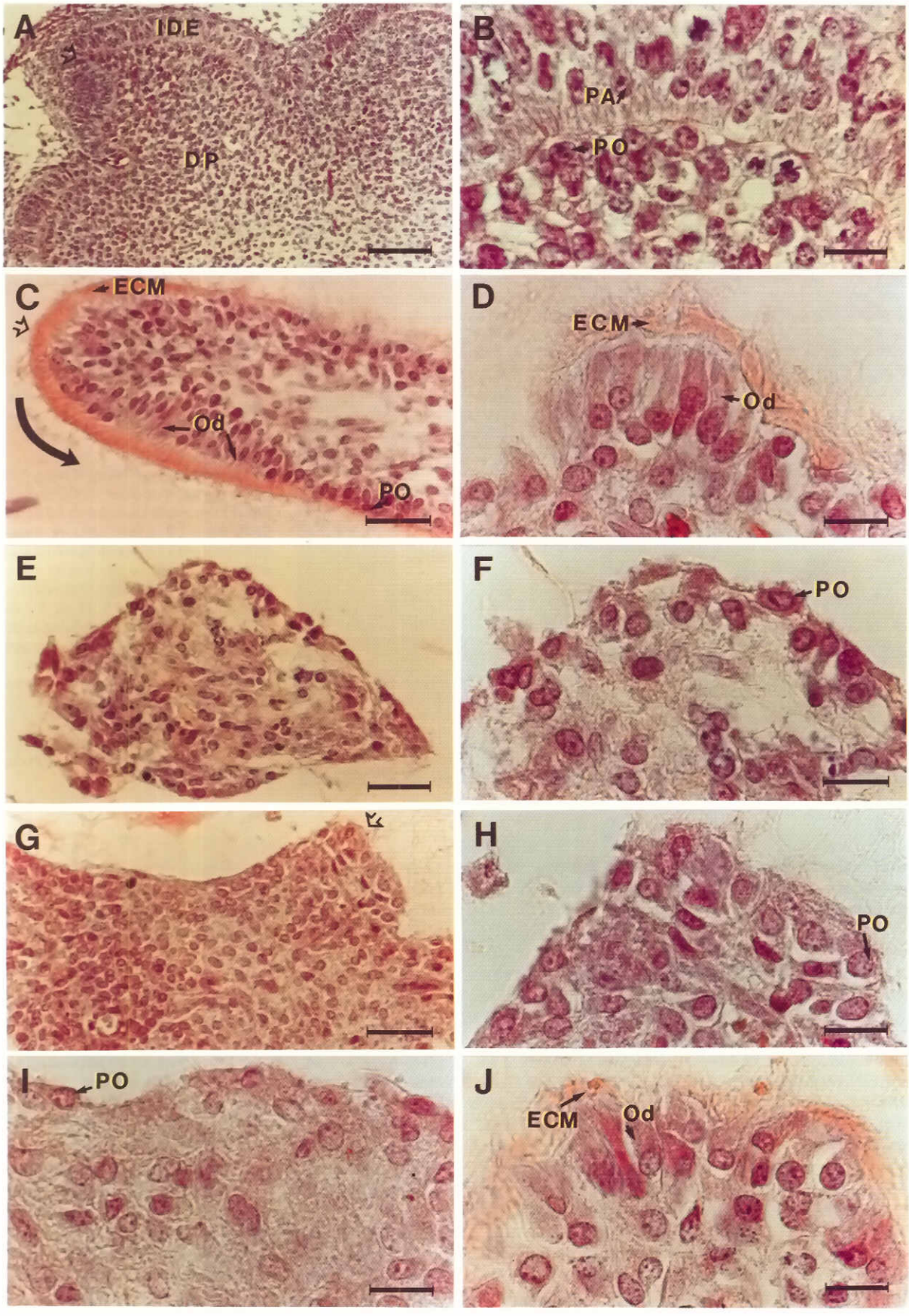
Heparin-agarose

When total EDTA-soluble dentin proteins were separated by chromatography on heparin-agarose (Fig. 5B), the fractions eluted with 0.15 M NaCl and 0.5 M NaCl were biologically active (Table 2): polarized and functional odontoblasts which deposited extracellular matrix were induced (Fig. 3J). These effects were observed on dental papillae cultured for 4 to 6 days (Table 2).

A similar study was carried out with dental papillae obtained from day-16 mouse embryos. The fractions which were active on day-17 dental papillae never promoted the differentiation on day-16 dental papillae even after 8 days of culture (data not shown).

Dental papillae cultured in the presence of BMP2 or TGFß1

Since the active fraction of the EDTA-soluble dentin proteins could be retained on heparin-agarose, BMP2 and TGFß1, which also have affinity for heparin and which are known to stimulate the


A
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metabolism of bone cells, were tested for their possible effects on cultured day-17 dental papillae.

BMP2 or TGFß1 alone

Initiation of odontoblast polarization never occurred when day-17 dental papillae were cultured in agar containing either TGFß1 alone or BMP2 alone (Fig. 6B,G respectively; Table 3). Since functional and cytological polarizations have never been experimentally dissociated, the general production of extracellular matrix induced by the two molecules at the periphery and within the whole dental papilla (Fig.6B,G) cannot be attributed to differentiated odontoblast-like cells. This matrix did not show the tubular structure characteristic of dentin. The increase in the production of extracellular matrix was more obvious when dental papillae were cultured in the presence of TGFß1 (50 ng per dental papilla) than in the presence of BMP2 (60 ng per dental papilla). In both types of culture, collagen type I (Fig. 7A,G) and fibronectin (Fig. 7B,H) were observed within the dental papillae cells.

The maintenance of cusp morphology was observed with higher frequency when dental papillae were cultured in the presence of TGFß1 than in the presence of BMP2. The effects of BMP2 were generally more discreet and seemed limited to the inside of the explant (Fig. 6).

BMP2 or TGFß1 combined with total EDTA-soluble dentin proteins

When dental papillae were cultured in the presence of either TGFß1 or BMP2 combined with non-active total EDTA-soluble dentin proteins, the cells at the periphery of the explant elongated, polarized and deposited extracellular matrix (Fig. 6D, H; Table 3). Collagen type I was a constituent of the matrix which accumulated at the periphery of the explant (Fig. 7C,I), but not fibronectin (Fig. 7D,J).

The most intense effect (87% of the cultured dental papillae demonstrated functional odontoblasts) was obtained with BMP2 (15 ng per dental papilla) combined with total EDTA-soluble dentin proteins (3.6 µg per dental papilla) (Fig. 6H; Table 3).

Polarization obtained in the presence of TGFß1 was more discreet (Fig. 6D). However, extracellular matrix was observed histologically within the whole dental papilla (Fig. 6D) under these conditions.

TGFß1 combined with heparin

A combination of TGFß1 with heparin also led to the initiation of odontoblast polarization (Fig. 6F; Table 3), while heparin alone had no effect on cultured dental papillae (Fig. 6E). After 6 days of culture the polarized odontoblasts were functional and secreted large amounts of extracellular matrix at their apical pole in contact with semi-solid medium (Fig. 6F). Only collagen type I was found to accumulate in contact with the semi-solid medium (Fig. 7E). Fibronectin was present in the whole dental papillae, without preferential accumulation (Fig. 7F). The dental papillae cultured in the presence of TGFß1 did not synthesize collagen type II.

Active fraction of dentin proteins with an antibody blocking TGFß activity

When added to the semi-solid medium enclosing one dental papilla, monoclonal 1D11.16-containing supernatant completely abolished the positive effects of the active fraction 5 obtained after DEAE-Cellulose separation. Polarized and functional odontoblasts observed in the presence of the active fraction (Fig. 8A) never appeared when cultured in the presence of the active fraction preincubated with the 1D11.16-containing supernatant (Fig. 8B). In these latter condition, round cells were seen without accumulation of extracellular matrix at the periphery of the explant (Fig. 8B); a very similar picture to that observed in the absence of proteins (Fig. 8C).

Possible influence of endogenous predentin on the differentiation of odontoblasts

Attempts were made to determine whether endogenous predentin secreted by differentiated odontoblasts could per se initiate the differentiation of neighboring preodontoblasts. The data reported in Table 4 showed that in cultured dental papillae there was a decrease in the surface area covered by differentiated odontoblasts relative to the corresponding controlateral intact molar. On the other hand the surface area covered by predentin was maintained or decreased to a lesser extent. However the volume of predentin increased dramatically in the cultured dental papillae implying the loss of control on odontoblast secretion.

Discussion

It is well known that demineralized bone matrix induces a sequence of events leading to cartilage and bone formation when implanted subcutaneously *in vivo* (Urist, 1965) and chondrogenesis in mesenchymal cells cultured *in vitro* (Seyedin *et al.*, 1983). Dentin proteins extracted by different protocols have also been shown to stimulate osteogenesis when implanted in muscles (Yeomans and Urist, 1967; Bessho *et al.*, 1990) and chondrogenesis (Harada *et al.*, 1990; Rabinowitz *et al.*, 1991) when added to the medium of several cultured mesenchymal cells. Using culture procedure described in this paper, EDTA-soluble dentin proteins separated on either DEAE-Cellulose or heparin-agarose columns were able to interact with dental mesenchymal cells located at the periphery of isolated dental papillae and initiate their terminal differentiation in odontoblasts. The chronology of these processes was similar to that of odontoblast differentiation in cultured day-17 tooth germs (Ahmad and Ruch, 1987). The absence of odontoblast differentiation, when dental papillae were cultured in the presence of the total EDTA-soluble fraction of dentin proteins, might result from a too low concentration of the active component(s).

Fig. 3. Sections of day-17 molars (A, B) and trypsin isolated dental papillae (C-J) in the presence (C, D, E, F, I, J) or in the absence of dentin proteins (G, H). *Day-17 mouse molars (A, B) consist of a dental papilla (DP) containing preodontoblasts (PO), separated from the inner dental epithelium (IDE) containing preameloblasts (PA) by a continuous basement membrane. In the presence of EDTA-soluble dentin proteins retained either on DEAE-Cellulose (C,D) or on heparin-agarose (J), preodontoblast differentiated: the cells became polarized and functional. Preodontoblasts never differentiated when dental papillae were cultured in the presence of the total EDTA-soluble dentin fraction (E, F), of proteins non-retained on heparin-agarose (I) or in the absence of proteins (G, H). Open arrows indicate the top of the cusps (A, C, G) and the solid arrow (C) the progressive differentiation of odontoblasts leading to a gradient. IDE, inner dental epithelium; DP, dental papilla; PO, preodontoblasts; Od, odontoblasts; ECM, extracellular matrix. Scale bars= 80 µm (A); 30 µm (C, E, G); 16 µm (B, D, F, H, I, J).*

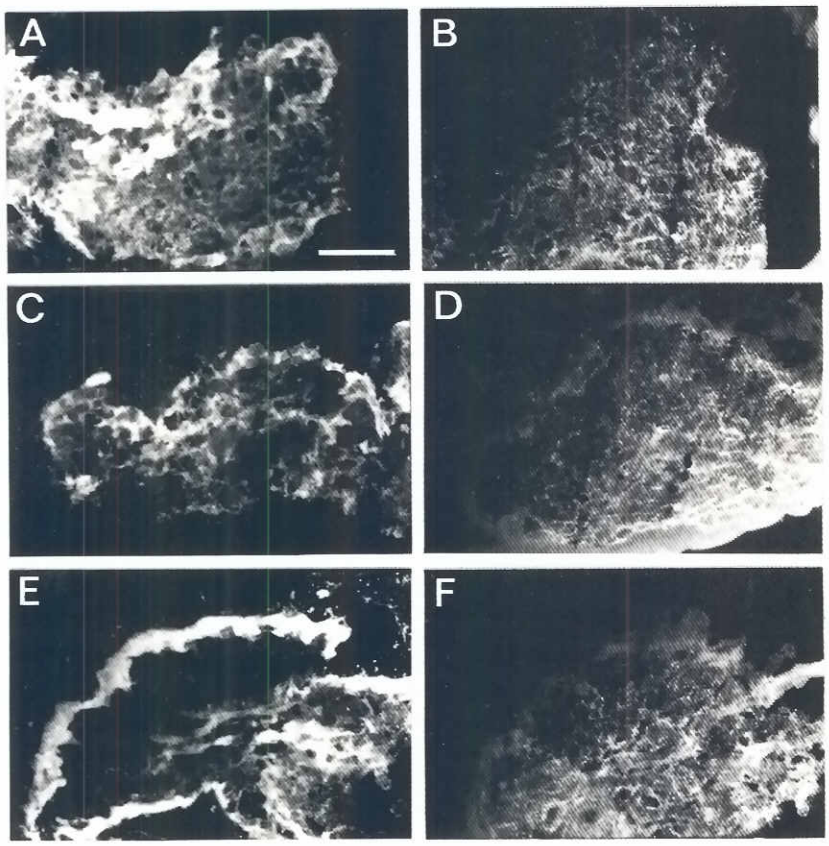

Fig. 4. Indirect immunofluorescence localization of collagen type I (A, C, E) and fibronectin (B, D, F) synthesized by isolated day-17 dental papillae embedded in semi-solid medium and cultured for 6 days. *Cultured dental papillae were embedded either in agar alone (A-B), or in agar containing proteins from the total EDTA-soluble fraction of dentin (C-D) or containing an active fraction of protein retained on DEAE-Cellulose (E-F). Odontoblasts which differentiated when cultured in the presence of dentin proteins retained on DEAE-Cellulose accumulated collagen type I at the periphery of the explant (E) but not fibronectin (F). Scale bar= 50 μm.*

Dentin has been reported to contain TGFß (Harada *et al.*, 1990), and the localization of TGFß1, TGFß2, TGFß3 and Vgr-1 transcripts in developing tooth germs suggests a possible coordinated role for the different transcription products during odontogenesis (Lehnert and Akhurst, 1988; Pelton *et al.*, 1989, 1990). Active fractions of the total EDTA-soluble dentin proteins in this study were retained on heparin-agarose and both TGFß1 (Rappolee *et al.*, 1988) and BMP2 (Wang *et al.*, 1988) also have affinity for heparin. For these reasons, attempts were made to compare the biological effects of our dentin proteins with those of TGFß1 and BMP2 and to determine whether in our system the activity of dentin proteins might be related to the presence of TGFß or other members of this supergene family of growth factors such as BMP2. BMP2A and TGFß1 are genetically related molecules (Wozney *et al.*, 1988), which have both been reported to stimulate osteogenesis (Joyce *et al.*, 1990; Yamaguchi *et al.*, 1991).

TGFß1 alone stimulated the secretion of both collagen type I and fibronectin without any cellular polarization. This observation agrees with previous reports where TGFß was described as a regulator of synthesis of extracellular matrix molecules such as collagen (Ignotz and Massagué, 1986) and fibronectin (Leonard *et al.*, 1991; Magnuson *et al.*, 1991; Wang *et al.*, 1991).

The effects of TGFß1 combined with heparin (which by itself has no effects) were qualitatively different: polarized cells secreted large amounts of extracellular matrix containing collagen type I at the periphery of the explant. Several reports have suggested possible synergistic interactions between the extracellular matrix and growth factors (for review see Schubert, 1992). Heparin has been reported to potentiate the activity of TGFß (McCaffrey *et al.*, 1989). In our conditions, the localized effect of TGFß1 in the presence of heparin might result from a decrease in the diffusibility

TABLE 1

POLARIZATION AND FUNCTIONAL DIFFERENTIATION OF ODONTOBLASTS IN THE PRESENCE OF DIFFERENT FRACTIONS OF DENTIN PROTEINS SEPARATED ON DEAE-CELLULOSE

Fractions	Duration of culture (days)	Number of cultured dental papillae	Dental papillae with polarized and functional odontoblasts
Total EDTA	6	6	0 (0%)
1	6	8	8 (100%)
2	6	6	5 (83%)
3	6	11	10 (90%)
4	4	10	9 (90%)
	6	8	6 (75%)
5	6	14	12 (85%)
Controls	6	14	0 (0%)

The total EDTA-soluble protein fraction of dentin or the different fractions separated by DEAE-Cellulose chromatography (Fig. 5A) were tested on day-17 mouse dental papillae embedded in semi-solid medium. Each dental papilla was cultured in the presence of 3.6 μg of proteins. Control cultures were performed in semi-solid medium without dentin proteins.

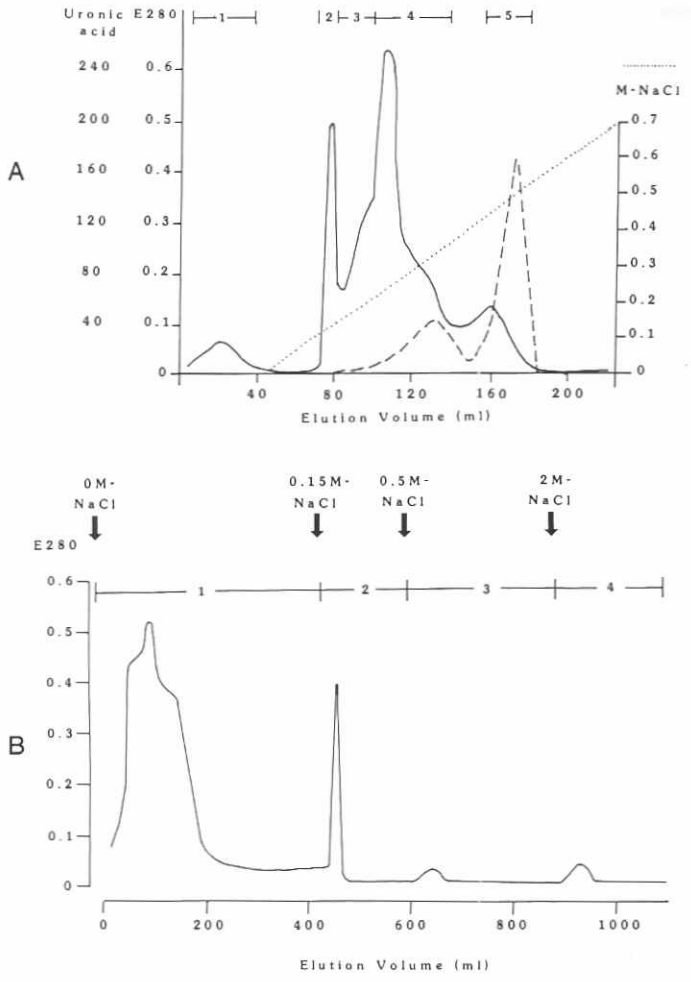

Fig. 5. DEAE-Cellulose chromatography (A) and heparin-agarose chromatography (B) of the total EDTA-soluble fraction of dentin proteins. (A) *After loading and washing of the DEAE-Cellulose column, a first peak of non-retained material was collected as fraction 1 and four other fractions (2-5) were then eluted in the presence of a linear gradient of NaCl (0 to 0.7 M).* **(B)** *During the loading and washing of the heparin-agarose column a first peak of non-retained material corresponding to the first fraction (fraction 1) was eluted and the other fractions were obtained after step-wise elution of the column by 0.15 M (fraction 2), 0.5 M (fraction 3) and 2 M (fraction 4) NaCl. Elution of proteins was monitored by measuring the absorbance at 280 nm. The potential biological activity of each individual fraction was tested on cultures of isolated dental papillae.*

of TGFß1. The biological effect of the active fractions of the total EDTA-soluble dentin proteins were very similar to those of the heparin-TGFß1 complex. This suggests that TGFß could bind to some unidentified constituent(s) of the dentin matrix. This hypothesis was further supported by the differentiation of odontoblast-like cells promoted by TGFß1 when associated with the inactive total EDTA-soluble fraction of dentin proteins. The nature of the dentin component(s) able to interact with TGFß1 will have to be investigated.

The inhibitory effect of 1D11.16 antibody on the differentiation of new odontoblast implies that TGFß1 or a related molecule is responsible for the initiation of odontoblast polarization induced by the active fractions of dentin proteins.

Dentin also contains BMP (Bessho *et al.*, 1991), and BMP2A mRNA has been detected in dental papillae cells including the odontoblast layer (Lyons *et al.*, 1990), but the related proteins have not been localized to date. More recently BMP2 messenger was found to be developmentally regulated (Rogers *et al.*, 1992), suggesting a possible role in mouse embryogenesis.

BMP2 and TGFß1 exhibit similar biological properties: by themselves both molecules stimulate matrix secretion in the whole explant while, when combined with inactive total EDTA-soluble dentin proteins, they can promote the cytological and functional differentiation of odontoblast-like cells. In this latter condition, BMP2 seems to be more active than TGFß1. However a larger number of specimens has to be examined to evaluate these differences. Nevertheless our observations suggest that both TGFß1 and BMP2 might bind to component(s) of the dentin matrix. These complexes might play a role in the induction of cytological and functional polarizations.

Cusp formation was already initiated in the first lower molar of day-17 dental papillae at the onset of culture. When embedded in agar and cultured in the presence of standard medium, the cusp pattern was maintained after 6 days of culture albeit with decreased size. When isolated day-17 dental papillae were cultured in agar containing active proteins, the initiation of odontoblast differentiation started at the tip of the cusps and then progressed through a gradient towards the basal parts as in intact teeth, i.e. the physiological gradients of differentiating odontoblasts were reproduced. The mechanisms responsible for these physiological gradients are still unclear, but a number of factors may be important. In intact teeth, odontoblasts are post-mitotic cells and several experimental studies including: 1) culture of heterochronal reassociations of dental tissues in the presence of ^{3}H-thymidine (Ruch *et al.*, 1976), and 2) *in vivo* and *in vitro* comparison of the kinetics of cell proliferation and cell differentiation (Ahmad and Ruch, 1987) have led to the hypothesis that the genetic programme of odontoblasts might determine a minimum number of cell cycles necessary to acquire the competence to answer to epigenetic signals triggering the terminal differentiation. This competence

TABLE 2

POLARIZATION AND FUNCTIONAL DIFFERENTIATION OF ODONTOBLASTS IN THE PRESENCE OF DIFFERENT FRACTIONS OF DENTIN PROTEINS SEPARATED ON HEPARIN-AGAROSE

Fractions	Duration of culture (days)	Number of cultured dental papillae	Dental papillae with polarized and functional odontoblasts
non retained	6	4	0 (0%)
0.15M NaCl	4	8	6 (75 %)
	6	8	6 (75%)
0.5M NaCl	6	9	8 (89%)

Three dentin protein fractions obtained after heparin-agarose chromatography (Fig. 5B) were tested on day-17 dental papillae embedded in semi-solid medium. Each dental papilla was cultured in the presence of 3.6 µg of proteins.

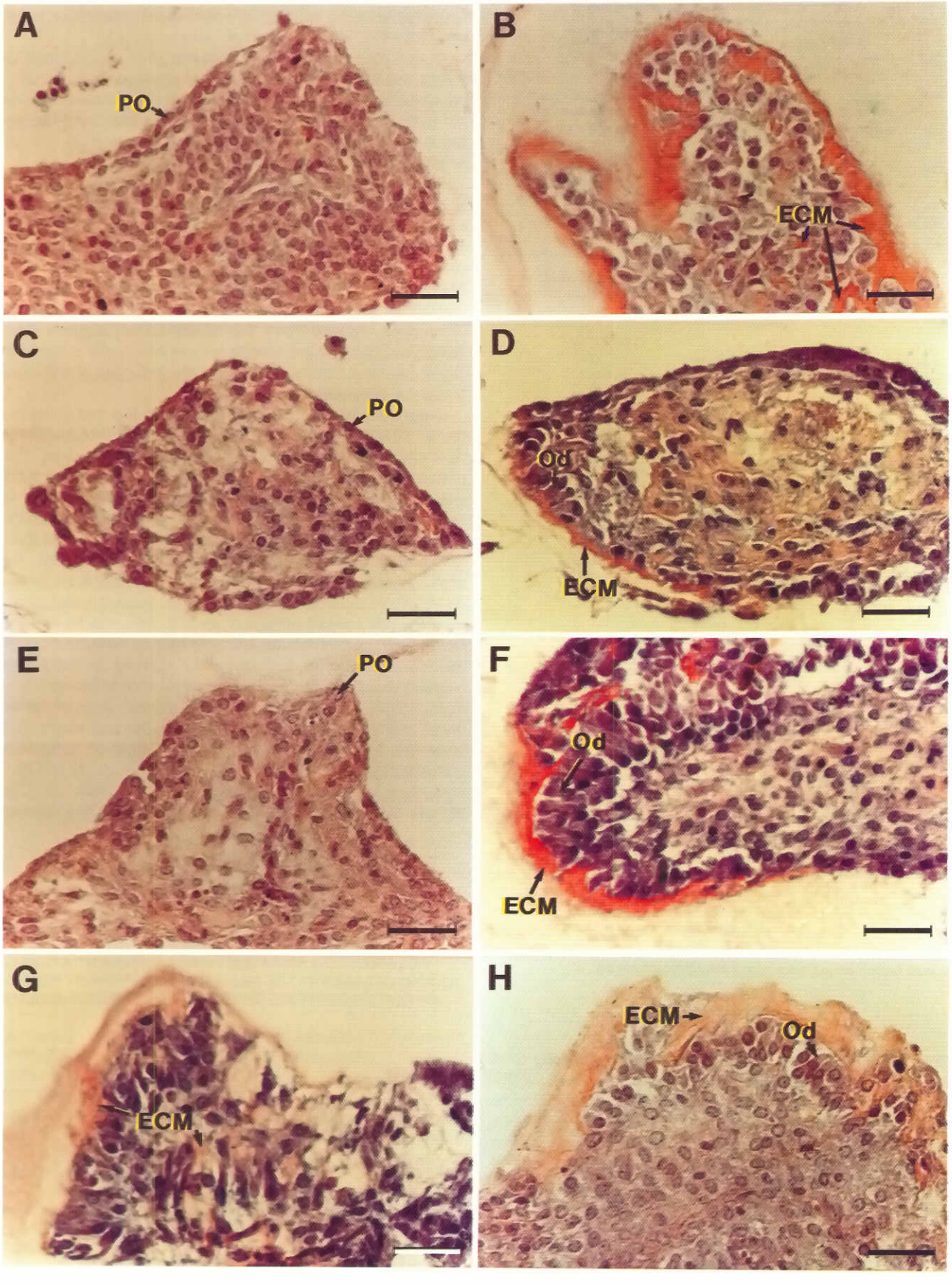
A
PO
B
ECM
C
PO
D
Od
ECM
E
PO
F
Od
ECM
G
ECM
H
ECM
Od

TABLE 3

POLARIZATION AND FUNCTIONAL DIFFERENTIATION OF ODONTOBLASTS IN THE PRESENCE OF MOLECULES OF THE TGFß SUPERFAMILY

	Dental papillae cultured in the presence of	Duration of culture (days)	Number of cultured dental papillae	Dental papillae with polarized and functional odontoblasts
rhBMP2	15 ng	6	5	0 (0%)
	60 ng	4	6	0 (0%)
		6	6	0 (0%)
rsTGFß1	10 ng	4	7	0 (0%)
		6	8	0 (0%)
	25 ng	4	11	0 (0%)
		6	9	0 (0%)
	50 ng	4	5	0 (0%)
		6	8	0 (0%)
rhBMP2 + (Total EDTA)	15 ng	6	8	7 (87%)
rsTGFß1 + (Total EDTA)	50 ng	6	9	5 (55%)
rs TGFß1 +	0 ng	6	7	0 (0%)
(heparin)	50 ng	6	9	5 (55%)

Different quantities of BMP2 and TGFß1 were tested alone or in combination with total EDTA-soluble fraction of dentin proteins (3.6 µg), and 50 ng of TGFß1 was also tested in combination with heparin (1.2 µg).

might arise, for example, by expression of cell-membrane constituents able to specifically interact with intrinsic matrix molecules or with paracrine growth factors (Ruoslahti and Yamaguchi, 1991; Schubert, 1992). These epigenetic signals may imply structural and/or compositional specificity of basement membrane components and/or matrix associated factors (Amar *et al.*, 1989). Furthermore the patterned distribution of differentiating odontoblasts probably involves a coordinated withdrawal from the cell cycle (Ruch, 1990).

The presence of gradients induced by dentin proteins might imply either the existence of odontoblast-preodontoblast cell-communication or both the maintenance of the epigenetic signals (active proteins) for several days in the agar and a progressive, autonomous and cell-kinetic-dependent acquisition of competence by further preodontoblasts. The first possibility could be ruled out for the following reasons: 1) our morphometric data shows that predentin, which should contain the active factors, allowed the maintenance of polarized odontoblasts and stimulated their secretion of matrix, but did not promote the progressive increase in the number of differentiated odontoblasts. 2) The continuous basement membrane, which covers EDTA-isolated day-18 dental papillae, allowed the initiation of functional differentiation of some odontoblasts at the tip of the main cusps *in vitro*, but did not allow the establishment of a gradient of differentiating cells (Osman and Ruch, 1981), i.e. the first differentiating odontoblasts did not promote the differentiation of their neighbors. A comparison of cell kinetics in intact teeth and isolated dental papillae will have to be performed to further clarify this point. As mentioned above, the first post-mitotic odontoblasts exist at the tip of the main cusps of day-18 first lower molars; it will be of importance to know if day-17 preodontoblasts undergo a last cell division before they can be triggered to overtly differentiate by dentin proteins. A disturbance in the kinetics of cell-proliferation could explain the absence of differentiated odontoblasts in cultured day-16 dental papillae.

The data presented in this paper demonstrate for the first time that in the presence of dentin matrix proteins and other growth factors, the initiation of odontoblast differentiation with maintenance of physiological gradients can be obtained, despite the absence of inner dental epithelium and a competent basement membrane.

Materials and Methods

Dentin extracellular matrix preparation

Total EDTA-soluble extracellular matrix proteins were prepared from rabbit incisor dentin in the presence of protease inhibitors as previously described (Smith and Leaver, 1979).

Lyophilized preparations of these matrix proteins were subjected to further purification by one of two methods: 1) DEAE-Cellulose chromatography, 2) heparin-agarose affinity chromatography.

DEAE-Cellulose chromatography

Samples (50 mg) of total EDTA-soluble dentin matrix proteins were applied to a column (18x1.0 cm) of DEAE-Cellulose (Whatman) equilibrated with 0.05 M Tris/HCl, pH 7.2. Pre-gradient material was eluted with the same buffer and further elution achieved with a salt gradient of 0-0.7 M NaCl in 0.05 M Tris/HCl, pH 7.2. Eluted material was monitored as previously described (Smith and Leaver, 1979) and pooled into the fractions 1-5 designated in Fig. 5A, before dialysis against distilled water and lyophilization. Fractions 1-5 correspond with the similarly numbered fractions used in the earlier study to examine the behavior of dental papillae cells when cultured in the presence of this material on Millipore filters in the absence of agar (Lesot *et al.*, 1986). Each isolated day-17 dental papilla, embedded in 12 µl of semi-solid medium containing 3.6 µg of proteins, was laid on Millipore filter put on 2.5 ml of liquid culture medium (Fig. 1).

Heparin-agarose affinity chromatography

Samples (100 mg) of the total EDTA-soluble dentin matrix proteins were also applied to a column (24 x 1.5 cm) of heparin-agarose (Promega) equilibrated with 0.05 M Tris/HCl, pH 7.2 containing deionized 6 M urea. After elution of the pre-gradient material with the same buffer, further elution was performed with step-wise increments of 0.15 M, 0.5 M, 2 M NaCl in the Tris/HCl urea buffer. Elution of proteins was monitored by absorbance at 280 nm and fractions corresponding to the total material eluted with each buffer pooled as designated in Fig. 5B. Protein fractions were dialyzed against distilled water and lyophilized and were added to the semi-solid medium in the same conditions as described above.

Fig. 6. Sections of day-17 dental papillae cultured for 6 days in semi-solid medium containing either TGFß1 alone (B), TGFß1 combined with total EDTA-soluble dentin proteins (D), TGFß1 combined with heparin (F), BMP2 alone (G) or BMP2 combined with total EDTA-soluble dentin proteins (H). *While odontoblasts never differentiated when cultured in the control medium (A), in the presence of total EDTA-soluble dentin proteins (C), or heparin (E), polarized cells were observed when TGFß1 or BMP2 was combined with total EDTA-soluble fraction of dentin proteins (D and H respectively) and when TGFß1 was combined with heparin (F). TGFß1 alone (B) only promoted a stimulation in extracellular matrix secretion. PO, preodontoblasts; Od, odontoblasts; ECM, extracellular matrix. Scale bar= 30 µm.*

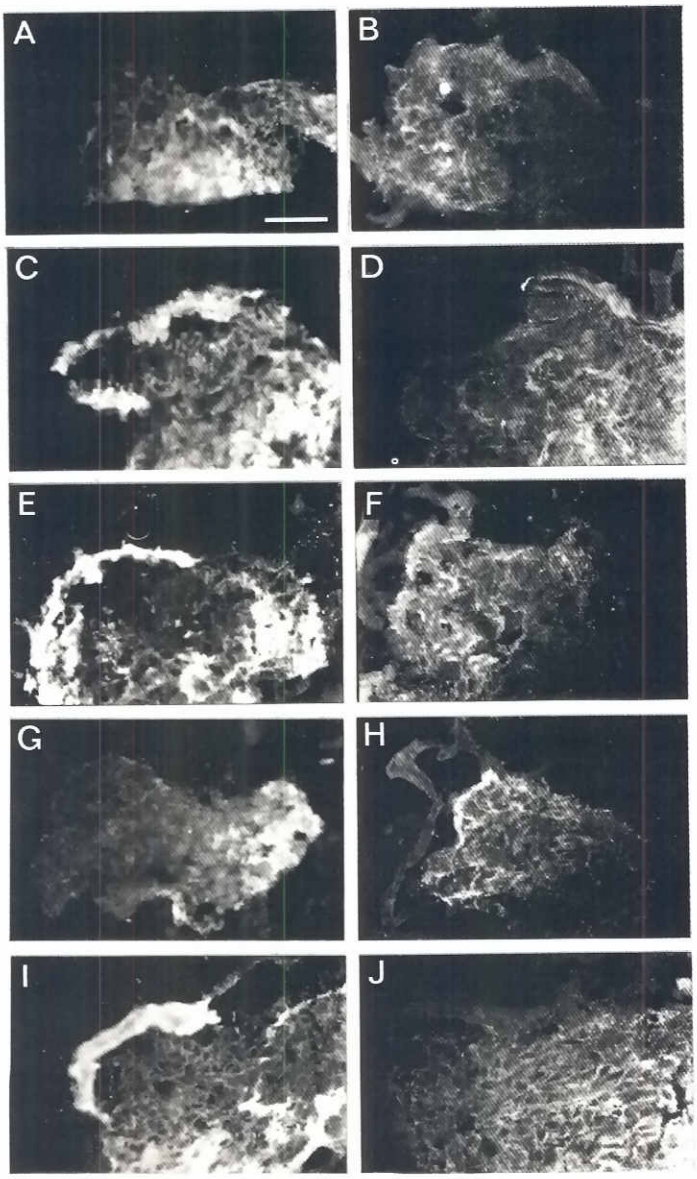

Fig. 7. Indirect immunofluorescent localization of type I collagen (A, C, E, G, I) and fibronectin (B, D, F, H, J) synthesized by day-17 dental papillae embedded in semi-solid agar medium and cultured for 6 days. *Cultured dental papillae were embedded in agar containing TGFß1 alone (A-B), TGFß1 and total EDTA-soluble dentin proteins (C-D), TGFß1 and heparin (E, F), BMP2 (G, H) or BMP2 and total EDTA-soluble dentin proteins (I, J). In conditions where odontoblasts could differentiate (C-D, E-F, I-J), collagen type I accumulated at the periphery of the explant, in front of differentiated cells (C, E, I) although fibronectin did not (D, F, J). Scale bar= 50µm.*

rs TGFß1

10 to 50 ng of recombinant simian Transforming Growth Factor ß1 (rsTGFß1: Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) were added to 12 µl of semi-solid medium embedding one day-17 dental papilla laid on a Millipore filter as described above.

3.6 µg of the dentin protein fraction number 5 obtained after the DEAE-Cellulose chromatography preincubated for 2 h at room temperature with 100 mg of a lyophilized monoclonal antibody-containing culture supernatant (1D11.16: Bristol-Myers Squibb Pharmaceutical Research Institute) were similarly added to the semi-solid culture medium. 1D11.16 has been shown to completely neutralize the bioactivities of TGFß1 and TGFß2 (Dasch *et al.*, 1989).

rhBMP2

15 and 60 ng of recombinant human Bone Morphogenetic Protein 2 (rhBMP2: Genetics Institute, Cambridge, MA) were added to the semi-solid medium as described for rsTGFß1.

Tooth germs

First lower molars of Swiss mouse embryos were removed either on day-16, day-17, or on day-18 of gestation (vaginal plug= day 0). The day-16 and 17 teeth contained dividing preameloblasts and preodontoblasts, whereas the day-18 teeth already contained the first polarized and post-mitotic odontoblasts at the top of the main cusps.

Trypsin dissociation

Enamel organs and dental papillae from day-17 tooth germs (Tyler stage 25) were enzymatically dissociated with 1% trypsin solubilized in Hanks' solution as previously described (Ruch *et al.*, 1976). Two passages in 1/1 fetal calf serum/Hanks' solution (V/V) were used to inhibit the activity of the enzyme.

Cultures of dental papillae in the presence of proteins

The newly dissociated day-17 dental papillae were deposited in a microwell containing 12 µl of a culture medium composed of RPMI-1640, vitamin-C (0.18 mg/ml), kanamycin (0.1 mg/ml), L-glutamin (2 mM), fetal calf serum (20%), and agar (0.5%). Cultures were performed in the presence of different proteins which were added at determined concentrations to the 12 µl of semi-solid medium. The different experiments involved culture of dental papilla with:

1) dentin matrix proteins: total EDTA-soluble proteins, DEAE-Cellulose or heparin-agarose fractions (3.6 µg),
2) BMP2 (15 to 60 ng),
3) TGFß1 (10 to 50 ng),
4) TGFß1 (50 ng) or BMP2 (15 ng) combined with total EDTA-soluble dentin proteins (3.6 µg),
5) TGFß1 (50 ng) combined with heparin (1.2 µg),
6) dentin matrix protein fraction 5 obtained after DEAE-Cellulose separation (3.6 µg) combined with 100 µg of lyophilized proteins of the 1D11.16-containing culture supernatant.

TABLE 4

COMPARISONS OF 1) THE SURFACE AREA COVERED BY ODONTOBLASTS 2) THE SURFACE AREA COVERED BY PREDENTIN AND 3) THE VOLUME OF PREDENTIN IN INTACT FIRST LOWER MOLARS OBTAINED FROM TWO EMBRYOS AT DAY 18 AND IN CONTROLATERAL ISOLATED DENTAL PAPILLAE CULTURED FOR 4 DAYS

	Odontoblasts area (mm^2)	Predentin area (mm^2)	Predentin volume (mm^3)
Tooth Germ TG	14.10^{-2}	$8.1.10^{-2}$	$2.0.10^{-4}$
Dental Papilla DP			
4 days of culture	$7.2.10^{-2}$	$6.3.10^{-2}$	$8.1.10^{-4}$
Ratio: DP/TG	0.51	0.77	4.00
Tooth Germ TG	$7.8.10^{-2}$	$3.4.10^{-2}$	$1.1.10^{-4}$
Dental Papilla DP			
4 days of culture	$5.4.10^{-2}$	$3.8.10^{-2}$	$5.2.10^{-4}$
Ratio: DP/TG	0.7	1.10	4.69

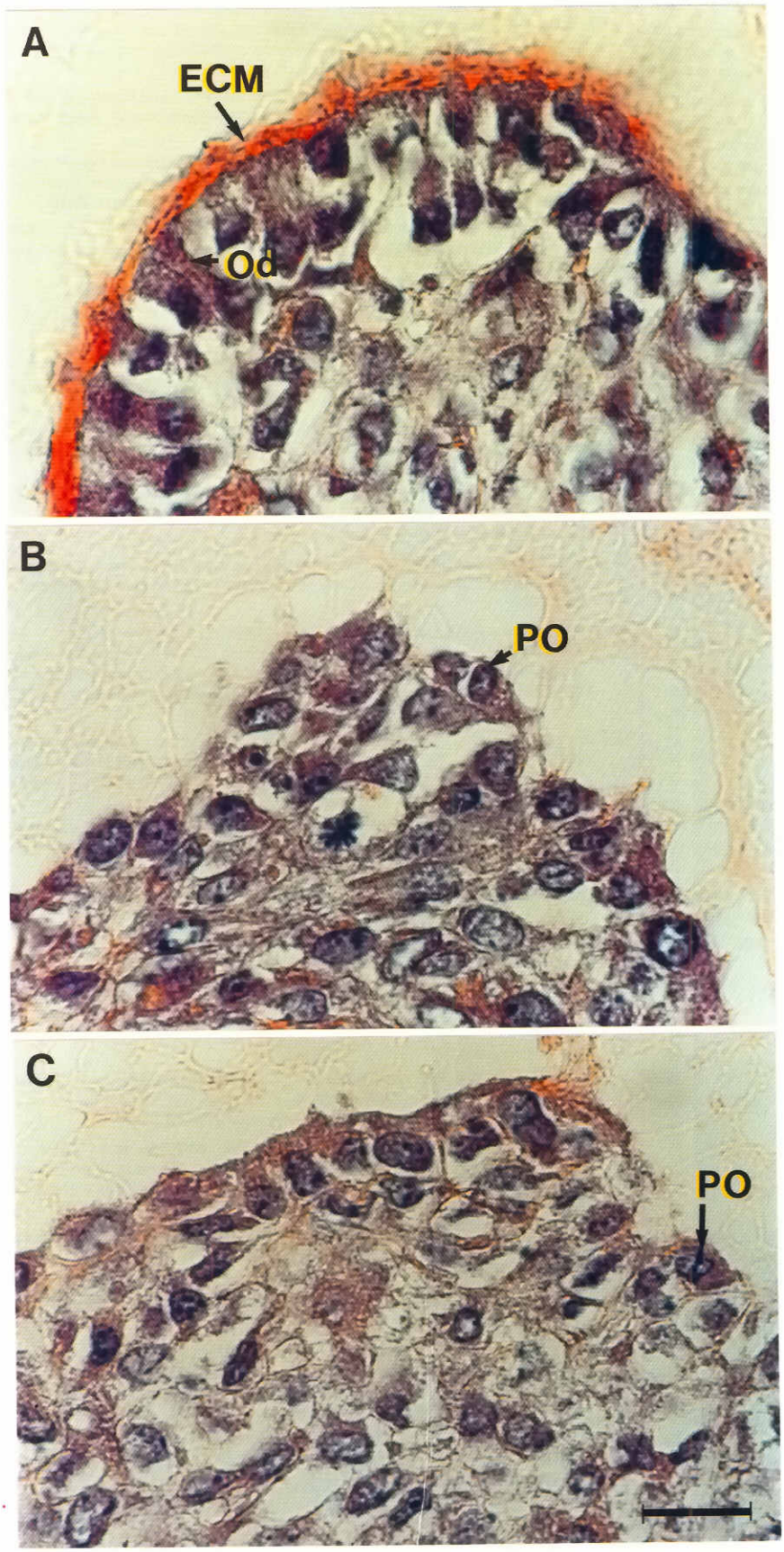

Fig. 8. Sections of day-17 dental papillae cultured for 6 days in semi-solid medium containing either an active fraction of dentin proteins, fraction 5 after DEAE-Cellulose (A), the same fraction preincubated with blocking antibody directed against TGFß (B). Control cultures were performed in the absence of proteins (C). *The polarization of peripheral cells, observed in the presence of the active fractions of dentin constituents (A) was completely abolished by the anti-TGFß antibody (B). PO, preodontoblasts; Od, odontoblasts; ECM, extracellular matrix. Scale bar= 16 µm.*

When proteins of different origins were combined a preincubation of 1 to 2 h was performed at room temperature in order to allow their interaction. The microtitration plate was placed on a thermostated table (37 to 40°C) to avoid solidification of the medium during dental papillae distribution. The preparation was then left for 10 min at 4°C and the embedded dental papillae were removed with the semi-solid medium and cultured for 6 days in Trowell-type cultures in 2.5 ml of medium comprising RPMI-1640, vitamin-C, kanamycin, L-glutamin, and fetal calf serum at the same concentrations as described above for the semi-solid medium. The successive steps are shown in the Fig. 1. The cultures were performed at 37°C in a humidified incubator under an atmosphere of 5% CO_2 in air and the culture medium was changed every two days.

Histology

Tissues were fixed in Bouin's solution. After paraffin-embedding, 5 µm thick-sections were cut and stained with Mallory's Alun haematoxylin.

Morphometric analysis

To assess the influence of endogenous predentin on odontoblast differentiation, morphometric analysis was performed. Intact day-18 first lower molars and controlateral isolated day-18 dental papillae cultured for 4 days in semi-solid medium according to Mark *et al.* (1990) were fixed in Bouin's solution, paraffin embedded and serial sections (7 µm) were analyzed. Three parameters were taken in account for morphometric measurements on each section: the length of the papilla surface covered by polarizing and functional odontoblasts, the length of the papilla surface covered by predentin, and the area of predentin. Because the plane of section can influence the appearance of odontoblast height leading to an underestimation of the area covered by polarized odontoblasts, the predentin area was also measured. The cumulated respective values allowed the calculation of 1) the surface covered by cytologically differentiated odontoblasts, 2) the surface area covered by predentin which corresponded to functional odontoblasts and, 3) the volume of predentin.

Immunofluorescence

Goat anti-rabbit fibronectin (Cappel Laboratories, Westchester, PA, USA), rabbit anti-mouse collagen type I (Andujar *et al.*, 1988) and rabbit anti-mouse collagen type II antibodies were used to stain frozen sections of cultured dental papillae.

Fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-goat immunoglobulins (Cappel Laboratories) and FITC-conjugated goat anti-rabbit immunoglobulins (Jackson, West Grove, PA, USA) were used as secondary antibodies.

Indirect immunofluorescence staining was performed as previously described (Lesot *et al.*, 1981, 1992).

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