Int. J. Dev. Biol. 36: 517-526 (1992)

Original Article

Effects of retinoids on tooth morphogenesis and cytodifferentiations, *in vitro*

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ABSTRACT The first embryonic lower mouse molar was used as a model system to investigate the effects of two retinoids, retinoic acid (RA) and a synthetic analogue, Ch55, on morphogenesis and cytodifferentiations *in vitro*. Exogenous retinoids were indispensable for morphogenesis of bud, cap and bell-stage molars in serum-free, chemically-defined, culture media. Transferrin and RA or transferrin and Ch55 acted synergistically in promoting morphogenesis from bud and cap-stage explants. Transferrin, *per se*, had no morphogenetic effect. Epithelial histogenesis, odontoblast functional differentiation and ameloblast polarization always occurred in RA-depleted explants. Comparison of the distributions of bromodeoxyuridine (BrdU) incorporation between explants cultured in the absence or presence of RA revealed that RA could modify the patterns of cell proliferation in the inner dental epithelium and dental mesenchyme. Inner dental epithelium cell proliferation is regulated by the dental mesenchyme through basement membrane-mediated interactions, and tooth morphogenesis is controlled by the dental mesenchyme. Laminin is a target molecule of retinoid action. Using a monospecific antibody, we immunolocalized laminin and/or structurally-related molecules sharing the laminin B chain in the embryonic dental mesenchyme and in the dental basement membrane and showed that RA could promote the synthesis or secretion of these molecules. Based on previous *in situ* hybridization data, it was speculated that CRABPs might regulate the effects of RA on embryonic dental cell proliferation. The fact that Ch55, a retinoid which does not bind to CRABPs, is 100 times more potent than RA in promoting tooth morphogenesis *in vitro* seems to rule out this hypothesis. On the other hand, the stage-specific inhibition of tooth morphogenesis by excess RA is consistent with the hypothesis that CRABPs might protect embryonic tissues against potentially teratogenic concentrations of free retinoids.

KEY WORDS: *odontogenesis, retinoic acid, transferrin, laminin, organ culture, chemically-defined culture medium*

Introduction

The embryonic tooth is an excellent tool with which to analyze the mechanisms of epithelial-mesenchymal interactions governing morphogenesis and cytodifferentiations. «Tooth morphogenesis» or «odontogenesis» commonly refers to the process whereby a local thickening of the oral epithelium, the dental lamina, together with a mass of condensing mesectodermal cells, transform progressively through successive steps (dental bud, cap and bell-stages) into an adult structure, the tooth crown, characterized by a definitive, highly specific, shape or «cusp pattern». Histogenesis of the dental epithelium, which takes place during the bell stage, gives rise to an enamel organ composed of four distinct layers: outer dental epithelium, stellate reticulum, stratum intermedium and inner dental epithelium (IDE). The latter, which encompasses the dental papilla mesenchyme, consists of preameloblasts. «Tooth cytodifferentiations» designate the mechanisms whereby the epithelial and mesenchymal cells localized at the interface give rise to highly specialized, terminally-differentiated secreting cells: the (mesenchyme-derived) odontoblast, secreting the constituents of predentin and dentin and the (epithelial-derived) ameloblast, secreting enamel-specific proteins (for reviews, see Ruch, 1984,

Abbreviations used in this paper: BCM, basal culture medium; BrdU, bromodeoxyuridine; CRABP, cellular retinoic acid-binding protein; ECM, extracellular matrix; EGF, epidermal growth factor; IDE, inner dental epithelium; PBS, phosphate buffered saline; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoid acid response element.

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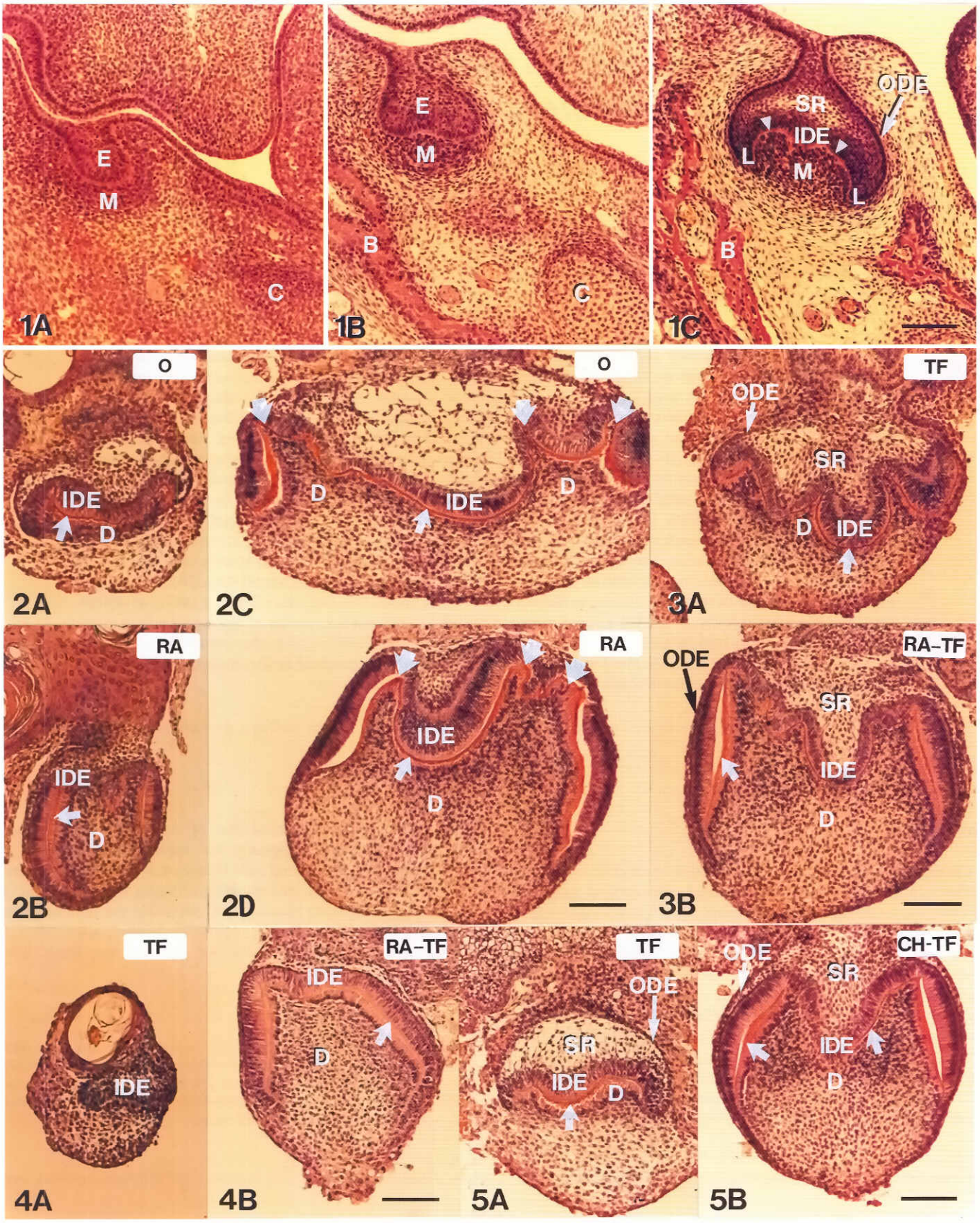
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1985, 1990; Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1983; Thesleff *et al.*, 1990, 1991). Within the framework of these definitions, tooth histo-morphogenesis and cytodifferentiations can be followed *in vitro*, eventually in serum-free, chemically-defined conditions (review: Slavkin *et al.*, 1989).

Retinoids exert profound effects on cell proliferation and differentiation, on histogenesis and pattern formation. For example, retinoids are necessary for normal histogenesis of skin and tracheal epithelia (Newton *et al.*, 1980; Asselineau *et al.*, 1989, 1992) in tissue cultures; they can re-specify anterior-posterior axis formation in the chick limb bud (for a review, see Eichele, 1989), mouse vertebral column (Kessel and Gruss, 1991) and *Xenopus* brain (Durston *et al.*, 1989). These effects of retinoids are thought to be primarily mediated by two distinct classes of nuclear receptors: the RARs and the RXRs. Ligand-activated RARs and RXRs act as transcriptional activators by binding to retinoid response elements (RAREs and RXREs) of target genes (for reviews, see De Luca, 1991; Glass *et al.*, 1991; Hashimoto and Shudo, 1991).

RARs and cellular retinoic acid-binding protein (CRABPs) transcripts have been detected in developing mouse teeth (Dollé *et al.*, 1990; Mark *et al.*, 1991). The embryonic tooth is a target organ of retinoid-induced teratogenesis *in vivo* (Knudsen, 1967 and references therein) and *in vitro* (Hurmerinta *et al.*, 1980) and vitamin A (retinol)-deficiency impairs tooth histogenesis *in vivo* (Mellanby, 1941; McDowell *et al.*, 1987 and references therein). However, there is no conclusive evidence for a role of retinoic acid (RA) during normal odontogenesis. In this study, we demonstrate that retinoids are indispensable for odontogenesis *in vitro* and unravel some of their mechanisms of action.

Results

RA is required for crown-morphogenesis of tooth germs cultured in a serum-free, chemically-defined culture medium

In preliminary experiments, serial concentrations of RA (1.5x 10^{-7}, 10^{-8}, 10^{-9}M) in basal culture medium (BCM) were tested for their ability to promote cusp formation from day-16 explants. The first two concentrations were found to be equally effective whereas the third had no significant effect. RA at $1.5x10^{-7}$ M was used throughout the study, unless otherwise specified in the materials and methods section; its effects depended on the developmental stage of the explanted tooth germ.

Day-14 molars are at the cap-stage (Fig. 1B). Explants cultured on BCM underwent histogenesis (Fig. 2A) and cytodifferentiations (Table 1, line h) but remained at the same apparent developmental stage as at the onset of the culture (i.e., cap stage; compare Fig. 2A with Fig. 1B). RA alone induced the down-growth of the cervical epithelial loop and triggered the formation of bell-shaped mini-teeth containing functional odontoblasts and polarized ameloblasts (Fig. 2B and Table 1, line h).

Day-16 molars are at the bell-stage and cusps have just begun to develop (Fig. 1C). Explants cultured on BCM demonstrated normal gradients of odontoblast and ameloblast differentiation. The absence of RA did not affect the timing of appearance of these terminally-differentiated cells (Table 1, lines j, k and l), but impaired normal cusp formation (Compare Fig. 2C and D).

The effects of RA and transferrin on early tooth development are synergistic

It has been previously demonstrated that transferrin, a serum protein, is required for early tooth development *in vitro* (Partanen *et al.*, 1984). Day-13 (bud-stage; Fig. 1A) and day-14 molars underwent morphogenesis only in BCM supplemented with transferrin and RA (Fig. 3B). Transferrin, by itself, promoted the growth of the explants (compare Fig. 3A with Fig. 2A) and made it possible to anticipate tooth cytodifferentiations (compare Table 1, lines d and e and lines f and g). Transferrin, *per se*, did not support morphogenesis: on transferrin-BCM (i.e., without RA) morphogenesis was stopped at the cap-stage and the IDE either remained flat or demonstrated erratic foldings (see for example Fig. 3A). The inhibitory effect of RA-deficiency on morphogenesis was already evident after 4 days in culture (not shown). On the other hand RA,

Fig. 1. Lower first molars at the onset of culture. *Frontal sections through the mandible of Swiss mouse fetuses at day-13* **(1A)**, *day-14* **(1B)** *and day-16* **(1C)**. *Prior to explantation, all mandibular bone (B) and Meckel's cartilage (C) were carefully removed.* **(1A)** *The day-13 molar is at the bud-stage. Note that the formation of mandibular bone has not yet been initiated.* **(1B)** *The day-14 molar has reached the cap-stage.* **(1C)** *The day-16 molar is at the bell-stage: histogenesis and cusp formation (arrowheads) have been initiated. E, dental epithelium, M, dental mesenchyme* **(1A,B)** *or dental papilla* **(1C)**; *ODE, outer dental epithelium, IDE, inner dental epithelium; SR stellate reticulum, L, cervical loop. Scale bar represents 100 μm.*

Fig. 2. Effect of RA on tooth morphogenesis in chemically-defined culture conditions. *Day-14* **(2A,B)** *and day-16* **(2C,D)** *molars were cultured respectively for 10 and 8 days on BCM* **(2A,C)** *or RA-supplemented BCM* **(2B,D)**. *RA induces the formation of bell-shaped mini-teeth* **(2B)** *or permits cusp formation* **(2D)**. *RA-depleted day-14 explants* **(2A)** *remain at the same apparent stage as at the onset of the culture. RA-deficient day-16 explants* **(2C)** *demonstrate hypoplastic cusps; also note that the odontogenesic mesenchyme encompassed by the IDE (i.e., dental papilla, D) is much reduced in these explants. Large arrows mark the tips of the cusps; small arrows, predentin; O, unsupplemented BCM; RA, RA-supplemented-BCM. Sagittal sections* **(2C,D)**. *Scale bar represents 100 μm.*

Fig. 3. Effects of transferrin (A) and transferrin plus RA (B) on morphogenesis of day-14 explants. *In the absence of RA* **(3A)** *transferrin promotes the growth of the explants (compare with Fig. 2A) but morphogenesis is not observed. IDE, inner dental epithelium; ODE, outer dental epithelium; SR, stellate reticulum; D, dental papilla; arrows, predentin; TF, transferrin-BCM; RA-TF, RA-supplemented transferrin BCM. Sagittal sections. Scale bar represents 100 μm.*

Fig. 4. Effect of RA on morphogenesis of trypsinized tooth explants. *Day-14 explants cultured for 4 days on transferrin-BCM* **(4A)** *or on RA-supplemented transferrin-BCM* **(4B)**, *trypsinized then cultured again for 4 days on the same media. IDE, inner dental epithelium; P dental papilla; arrows, predentin; TF, transferrin-BCM; RA-TF, RA-supplemented transferrin-BCM. Scale bar represents 100 μm.*

Fig. 5. Effect of Ch55 on tooth morphogenesis. *Day-13 explants were cultured for 10 days on transferrin-BCM* **(5A)** *and on the same medium but supplemented with Ch55* **(5B)**. *Note that the histogenesis of the enamel organ proceeds normally in the absence of retinoid. IDE, inner dental epithelium; ODE, outer dental epithelium; SR, stellate reticulum; D, dental papilla; arrows, predentin; TF, transferrin-BCM; CH-TF, Ch55-supplemented transferrin-BCM. Frontal sections. Scale bar represents 100 μm.*

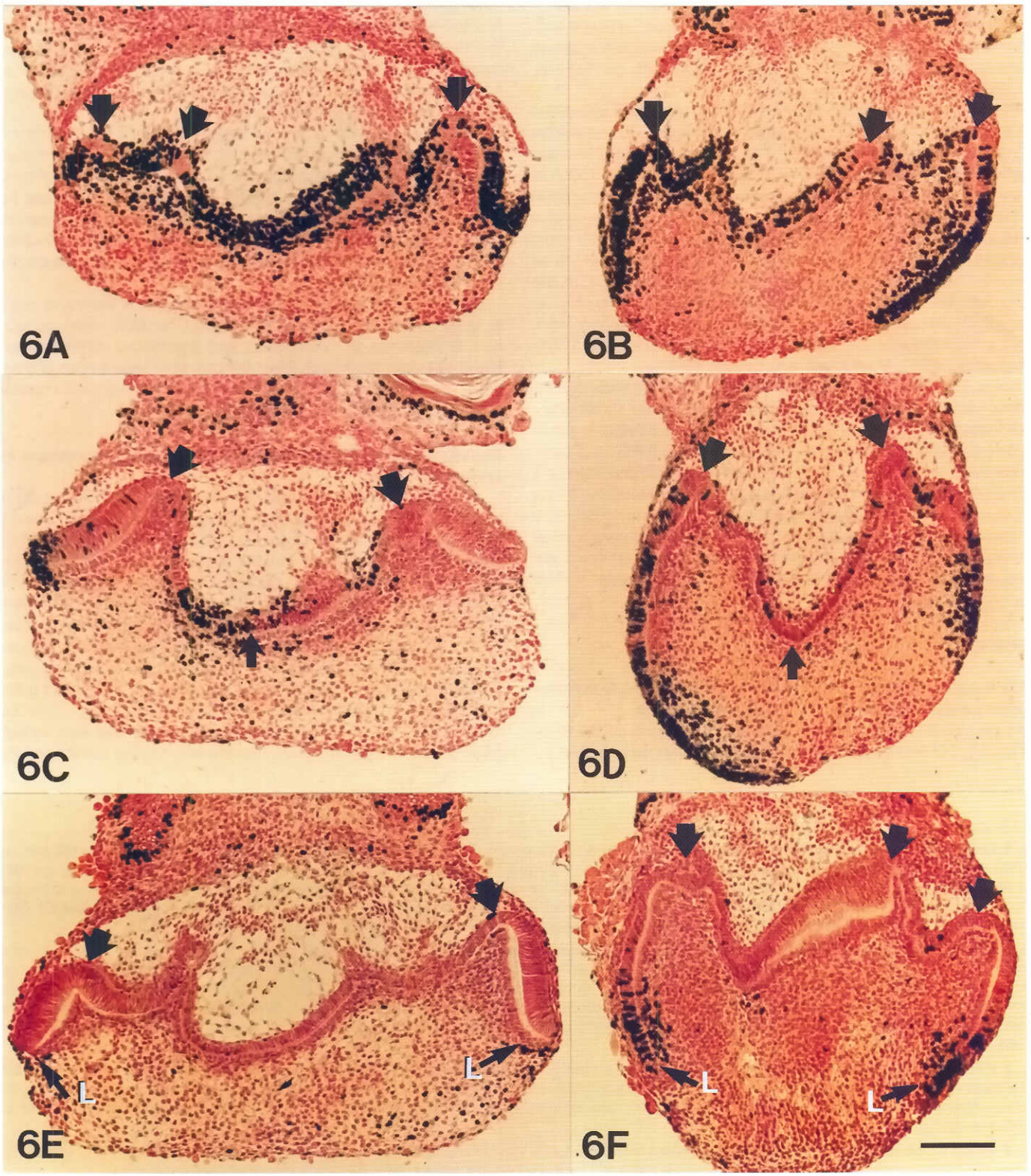

Fig. 6. Effect of RA-deficiency on cell proliferation patterns. *Sagittal sections through day-16 molars incubated in the presence of BrdU after 4* **(6A, B)**, *6* **(6C,D)** *and 7* **(6E,F)** *days in culture, in BCM* **(6A,C,E)** *or RA-supplemented BCM* **(6B,D,F)**. *Nuclei which have incorporated BrdU are stained in black. Note the persistence of the labelling over the intercuspal region (small arrows) and its disappearance from the cervical loop regions (L) after 6* **(6C)** *and 7* **(6E)** *days in culture in RA-depleted explants. Large arrows, cusps. Sagittal sections. Scale bar represents 100 μm.*

per se, did not affect the timing of appearance of odontoblasts and ameloblasts (Table 1, lines a-h and j-l). The absence of RA did not impair epithelial histogenesis from bud-stage molars explanted on day-13 or dissected from cultured day-11 mandibular arches (Table 1, lines a-c and data not shown).

Day-14 trypsinized explants maintained on RA-supplemented transferrin-BCM underwent morphogenesis and cytodifferentiations (Fig. 4B and Table 1, line i). Such explants progressively regressed in the absence of RA (Fig. 4A and Table 1, line i).

RA and Ch55 exert identical morphogenetic effects on molar explants

Ch55 is a synthetic retinoid which binds to RARs as efficiently as RA but has no binding affinity for CRABPs (review: Hashimoto and Shudo, 1991). In preliminary experiments serial concentrations of Ch55 ($1.5x10^{-9}$, 10^{-10}, 10^{-11} M) were tested for their ability to promote cusp morphogenesis from day-16 explants. The first concentration prevented cusp formation, the third was ineffective. Ch55 at $1.5x10^{-10}$ M improved tooth morphogenesis (compare Fig. 5A and B) similarly to RA at $1.5x10^{-7}$ M or $1.5x10^{-8}$ M.

RA deficiency alters the patterns of cell proliferation in the IDE and dental mesenchyme

Differential mitotic activities of the IDE, controlled by the dental mesenchyme have been correlated with molar crown morphogenesis (Olive and Ruch, 1982; Ruch, 1990). Possible effects of RA on cell proliferation were investigated by comparing the distribution of BrdU incorporation between day-16 explants cultured on RA-supplemented and unsupplemented BCM. A 20 h labeling by BrdU corresponds to the mean duration of the cell cycle in both epithelial and mesenchymal compartments of these explants (Ahmad and Ruch, 1987). Thus, this approach makes it possible to distinguish cycling cells from those that are already post-mitotic when BrdU is added to the culture medium. After 4 days, the first post-mitotic epithelial cells were observed at the tip of the forming cusps both on BCM and RA-supplemented BCM (Fig. 6A, B).

Molars cultured for 6 days without RA consistently showed strong anti-BrdU labeling of the intercuspal IDE (Fig. 6C), whereas such a labeling was weak or absent in molars cultured in the presence of RA (Fig. 6D). On the other hand, after 7 days in culture, labeling of the cervical epithelial loop and adjacent dental mesenchyme was consistently observed only on RA-supplemented BCM (compare Fig. 6E and F).

Laminin is synthesized by dental mesenchymal cells and its expression is up-regulated by RA

Sections from day-14 molars cultured for 4 days demonstrated strong anti-laminin immunostaining in the basement membrane but also in the dental papilla including the layer of preodontoblasts (Fig. 7). However, there was no significant difference in the pattern or intensity of immunofluorescence between RA-supplemented and RA-deprived explants (not shown). Such differences became conspicuous in explants that were recultured following trypsinization.

In day-14 explants maintained in RA-supplemented BCM, the basement membrane was uniformly labeled by anti-laminin antibodies after 2 days (Fig. 8B). In RA-depleted, trypsinized explants, laminin deposition was impaired (Fig. 8A). As already mentioned, such explants regressed. In another set of experiments, day-16 molars were cultured for 4 days in BCM, then treated with trypsin. Half of the trypsinized explants were then cultured for 30 h in the presence of RA, whereas the other half, consisting of the contralateral molars, was returned to BCM for the same period. RA promoted the deposition of laminin in the basement membrane and dental papilla ECM (Compare Fig. 8C and D). Sections incubated with non-immune rabbit serum were not labeled (example Fig. 8E).

TABLE 1

EFFECTS OF RETINOIDS AND TRANSFERRIN ON ODONTOBLAST DIFFERENTIATION AND AMELOBLAST POLARIZATION

Initial stage (days)	Days in culture	Medium/N° of explants	Explants with odontoblasts	Explants with predentin	Explants with ameloblasts	
11	12	TF-BCM/6	6	5	2	a
		RATF-BCM/8	8	8	5	
13	8	TF-BCM/10	5	1	0	b
		Ch55TF-BCM/10	6	1	0	
	10	TF-BCM/7	7	7	6	c
		Ch55TF-BCM/7	7	7	7	
14	6	BCM/8	0	0	0	d
		RA-BCM/8	0	0	0	
		TF-BCM/7	7	3	1	e
		RATF-BCM/7	6	3	0	
	8	BCM/7	3	2	0	f
		RA-BCM/7	2	0	0	
		TF-BCM/10	10	10	8	g
		RATF-BCM/10	10	10	9	
	10	BCM/6	6	6	6	h
		RA-BCM/8	8	8	8	
	4+4*	TF-BCM/7	1	1	1	i
		RATF-BCM/7	7	7	7	
16	4	BCM/10	0	0	0	j
		RA-BCM/10	0	0	0	
	6	BCM/10	10	10	2	k
		RA-BCM/9	9	9	1	
	8	BCM/10	10	10	10	l
		RA-BCM/11	11	11	10	

TF-BCM, transferrin-BCM; RA-BCM, RA-supplemented BCM; RATF-BCM and Ch55TF-BCM, RA and Ch55-supplemented transferrin-BCM. *Teeth cultured for 4 days, trypsinized then recultured.

The inhibitory effects of excess RA on tooth morphogenesis are stage-dependent

The effects of excess RA were studied on embryonic molars cultured on serum-supplemented medium.

In explants from day-14, cusp morphogenesis is initiated after 4 days in culture in the absence of added RA (Mark *et al.*, 1990). At $0.7x10^{-5}$ M RA morphogenesis always proceeded to the bell-stage but cusp formation was inhibited (compare Fig. 9A and B). Day-16 molars cultured in the presence of $0.7x10^{-5}$ M RA assumed a monocuspal aspect (Fig. 9C).

Discussion

Retinoids are indispensable for tooth morphogenesis in serum-free, chemically-defined media during the bud, cap and bell-stages.

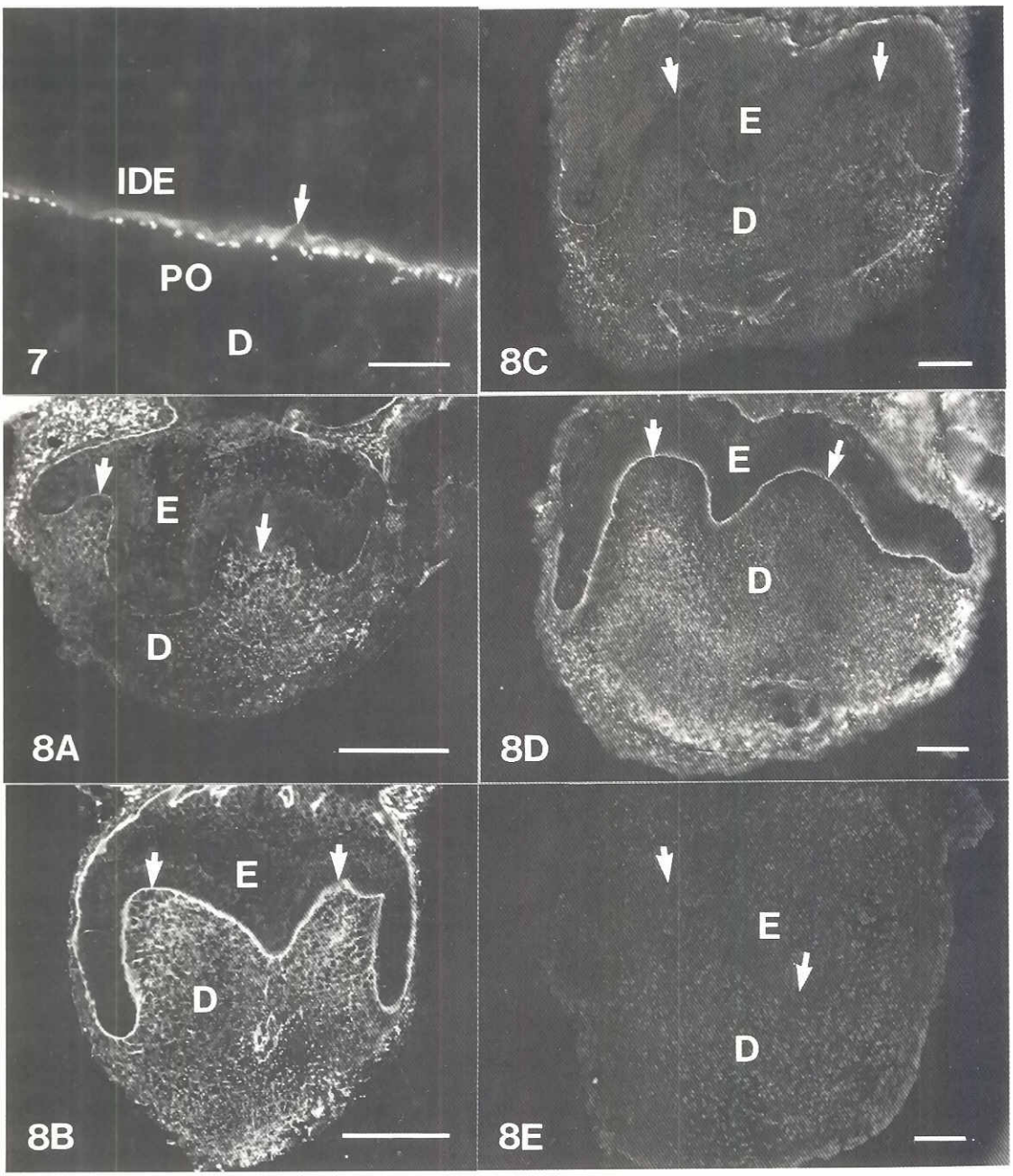

Fig. 7. Immunofluorescence localisation of laminin in a day-14 explant cultured for 4 days on transferrin-BCM; *high magnification of the epithelial-mesenchymal interface: note the presence of immunofluorescent spots in the preodontoblast layer (PO). IDE, inner dental epithelium; arrow, basement membrane, D, dental papilla. Scale bar represents 15 μm.*

Fig. 8. Effect of RA on laminin deposition in trypsinized tooth explants. (8A,B) *Day-14 explants cultured for 4 days on transferrin-BCM* **(8A)** *or RA-supplemented transferrin-BCM* **(8B)** *then trypsinized and cultured again for 2 days in the same media.* **(8C,D)** *Day-16 explants cultured for 4 days on BCM then trypsinized and cultured again for 30 h on BCM* **(8C)** *or on RA-supplemented-BCM* **(8D)**: *RA promotes the deposition of laminin in the basement membrane (arrows) and dental papilla (D).* **(8E)** *Explant similar to* **(8D)**: *the section was incubated with non-immune rabbit serum. E, enamel organ. Indirect immunofluorescence. Scale bars represents 50 μm.*

RA or Ch55 alone promoted cusp formation from bell-stage explants. RA, by itself, induced the transformation of the dental cap into a bell conformation. Retinoids and transferrin were both required for morphogenesis of bud and cap-stage explants. In contrast, RA-depletion had no effect on odontoblasts and ameloblast terminal differentiation: in the absence of retinoids, odontoblast polarization was not delayed and these cells always became functional. Furthermore, ameloblast polarization, which requires the presence of

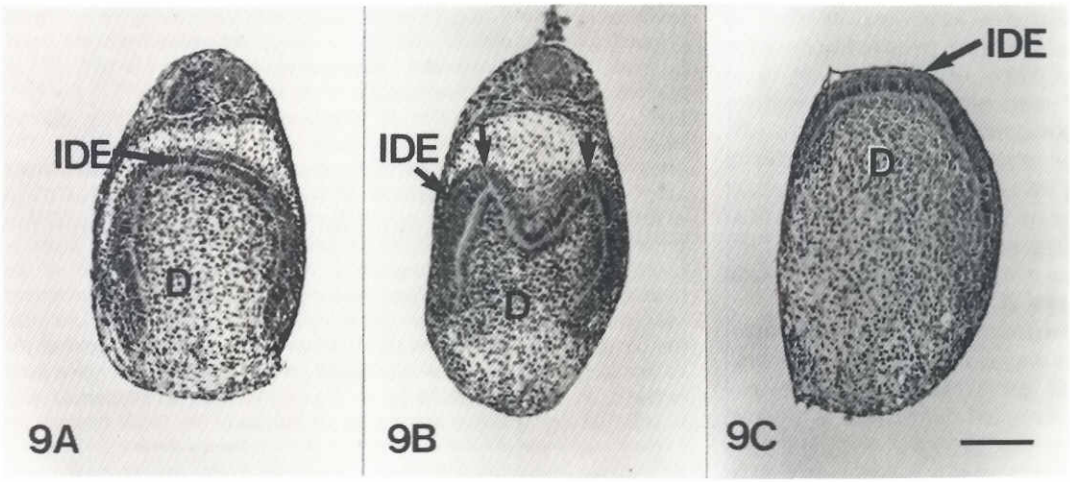

Fig. 9. Effect of excess RA on tooth morphogenesis. *Day-14* **(9A,B)** *or day-16* **(9C)** *molars cultured for 6 days on serum-supplemented medium in the presence of 0.7 x 10^{-5} M RA* **(9A,C)** *or in the absence of RA* **(9B)**. *Excess RA specifically inhibits cusp formation. IDE, inner dental epithelium; D, dental papilla; arrows, cusps. Scale bar represents 100 μm.*

predentin (Karcher-Djuricic *et al.*, 1985) always occurred within 2 days after the onset of predentin secretion. Likewise, the histogenesis of the dental epithelium seems to be retinoid-independent. It is interesting to note that the same low concentrations of retinoids which promoted morphogenesis of day-13 molars consistently inhibited bone formation from osteoprogenitor cells located at the periphery of these explants (results not shown). This implies that the local concentrations of free retinoids should be tightly regulated, *in vivo*.

The iron-transporting serum glycoprotein, transferrin, acts as a «growth factor» during development (Ekblom *et al.*, 1981, 1983). Transferrin is essential to early molar development *in vitro*, but it is no longer required for culturing molars that have reached the bell-stage at the time of explantation (Partanen *et al.*, 1984). From this stage on, transferrin requirements are apparently satisfied by endogenous transferrin retained in the explant (Partanen and Thesleff, 1987a). We found that transferrin by itself promoted the growth of bud and cap-stage molars and that transferrin-deficiency delayed odontoblast terminal differentiation. These observations are consistent with previous data by our group demonstrating that transferrin stimulates the *in vitro* proliferation of pre-odontoblasts and pre-ameloblasts (Cam *et al.*, 1989) and that odontoblast terminal differentiation can only occur after a minimal number of cell cycles (Ruch *et al.*, 1982). Transferrin *per se* had no morphogenetic effect. RA and transferrin acted synergistically in promoting the formation of the dental bell and/or the development of cusps.

Crown morphogenesis, which is controlled by the dental mesenchyme, implies, among other phenomena, differential mitotic activities of the IDE (Olive and Ruch, 1982; Ruch, 1990). RA-deficiency affects the patterns of cell proliferation in cultured mouse molars: in the absence of RA, the withdrawal from the cell cycle is delayed in the intercuspal region but anticipated in the cervical loop region. Candidate target genes for mediating the action of retinoids on cell proliferation-dependent crown morphogenesis include: growth factors and growth factor receptor genes, transcriptional factor genes, genes coding for ECM macromolecules and ECM degrading enzymes (Desbois *et al.*, 1991 and references therein; Glass *et al.*, 1991; Hashimoto and Shudo, 1991) and integrin genes (Rossino *et al.*, 1991).

During odontogenesis *in vivo*, transferrin receptors are preferentially located in areas of active cell proliferation such as the cervical loop region (Partanen and Thesleff, 1987b). Transferrin and RA both affect cell proliferation. Thus tooth morphogenesis represents a useful developmental model to investigate possible functional interferences between activation of RARs by retinoids and transferrin receptor expression (Ho *et al.*, 1989). Embryonic molars also express EGF receptors (Partanen and Thesleff, 1987c; Cam *et al.*, 1990) and EGF receptor expression in the embryonic tooth is altered by exogenous RA (Abbott and Pratt, 1988). Recent findings demonstrate that, depending on the cell type, ligand-activated RAR could up-or-down-regulate the transcription of the EGF receptor (for a review, see Glass *et al.*, 1991). These data are interesting with respect to the effects of EGF on odontogenic cell proliferation *in vitro*: EGF uncouples cell proliferation kinetics in epithelial and mesenchymal cells, thereby inhibiting morphogenesis (Partanen *et al.*, 1985).

There is considerable experimental evidence from tissue cultures that tooth morphogenesis is controlled by the dental papilla (Kollar and Baird, 1969, 1970) and that IDE cell proliferation is regulated by the dental papilla through basement membrane-mediated interactions (Olive and Ruch, 1982).

Laminin, a large glycoprotein of basement membranes, might play an important role in embryonic epithelial-mesenchymal interactions (Schuger *et al.*, 1990, 1991). It consists of three genetically distinct polypeptide chains (A, B1 and B2). The promoter region of the laminin B1 chain gene contains a RARE (Vasios *et al.*, 1989, 1991). It was recently found that laminin B chains are expressed in a variety of mouse embryonic mesenchymal matrices which all lack the A chain (Klein *et al.*, 1990; Simo *et al.*, 1991), in addition to basement membranes. Furthermore the B chain of basement membrane-laminin is produced by both epithelial and mesenchymal cells in the developing intestine (Simo *et al.*, 1992). In previous studies (Lesot *et al.*, 1981; Thesleff *et al.*, 1981), laminin was immunolocalized in the basement membranes of the developing tooth; within the dental papilla, it was found only in capillary basement membranes. The monospecific, affinity-purified antibodies used in the present study made it possible to visualize anti-laminin immunoreactivity, most probably corresponding to B chains (see above), in the dental papilla proper including the apical pole of preodontoblasts. This indicates that part of the basement mem-

brane laminin found at the epithelial-mesenchymal interface is synthesized by the mesenchyme. Following trypsinization, which destroys the extracellular laminin (Lesot *et al.*, 1981), RA could rapidly promote the deposition of newly synthesized laminin in the basement membrane between epithelium and mesenchyme and in the ECM of the dental papilla. Our data do not make it possible to determine whether the effects of RA on laminin synthesis and/or secretion are direct or secondary to modifications of the overall ECM organisation, but they strongly suggest that the regression of RA-depleted, trypsinized explants is causally related to their inability to produce or assemble an appropriate ECM.

In a previous study we have shown that during tooth development, CRABPI and CRABPII transcriptions are respectively associated with mesenchymal and epithelial cells exhibiting high levels of cell proliferation. Our present data reinforce the idea that areas of active cell proliferation are indeed preferential targets of retinoid action. The demonstration that Ch55, a retinoid which does not bind to CRABPs, is 100 times more potent than RA in promoting tooth morphogenesis argues against a qualitative role for CRABPs in the control exerted by retinoids on odontogenesis *in vitro*. Using a similar approach, Asselineau *et al.* (1992) reached the same conclusion for skin histogenesis *in vitro*. Moreover it has been shown that synthetic retinoids with the property of binding to RARs but not to CRABPs are able to induce limb duplications in the chick limb bud (Maden *et al.*, 1991). It has also been hypothesized that CRABP expression by embryonic tissues might protect them against potentially teratogenic concentrations of free retinoids. We found that RA in excess prevented molar morphogenesis in a stage-specific manner: it did not affect the transformation of the dental cap into a bell conformation but inhibited the subsequent step of morphogenesis, i.e., cusp formation. First lower molars at the cap-stage demonstrate high levels of CRABP II transcription throughout their IDE. During the bell-stages, CRABP II transcription is restricted to the cervical loop IDE (Mark *et al.*, 1991). Mesenchyme-specific transcription of CRABPI is not detected at the cap-stage: according to the above-mentioned hypothesis, the target tissue of retinoid teratogenicity is the epithelium. Thus changes in the expression patterns of CRABPII transcripts might account for the apparent increase in sensitivity of dividing IDE cells towards excess RA as development proceeds from the cap to the bell-stage.

Materials and Methods

Organ culture in serum-supplemented medium

Lower first molars were dissected from Swiss mouse fetuses killed on days-14 or 16 (vaginal plug = day 0). The tooth germs were cultured for 6 days either in the absence or presence of RA (all-trans RA, Sigma), on a semi-solid medium, as previously described (Mark *et al.*, 1990). This medium, consisting of RPMI-1640 supplemented with 20% fetal calf serum, L-glutamine (2 mM), kanamycin (100 μg/ml) and ascorbic acid (180 μg/ml) was gelled by 0.5% Agar. RA dissolved in absolute ethanol in 500 times stock solutions was added to the culture medium to a final concentration of 0.7×10^{-5} M. This concentration was initially chosen according to a previous report by Hurmerinta *et al.* (1980). Control cultures received the same volume of ethanol. Cultures were kept in the dark and the medium changed every other day.

Organ culture in serum-free, chemically-defined media

Day-13, 14 and 16 first lower embryonic molars were cultured for 4-12 days in the same medium as above but without fetal calf serum. This medium is referred to as basal culture medium (BCM). The explants from day-14 and 16 were obtained almost free of non-dental mesenchyme, whereas those from day-13 were dissected with surrounding mesenchyme containing (among others) presumptive osteoblast precursors of the alveolar bone. BCM was eventually supplemented with RA (1.5×10^{-7}, 10^{-8} or 10^{-9} M) or with serial concentrations of the RA analogue Ch55 (1.5×10^{-9}, 10^{-10}, 10^{-11} M; Kagechika *et al.*, 1989) and/or with transferrin (human transferrin, iron saturated, cell culture tested, Sigma; 50 μg/ml). The retinoids were added to BCM or to transferrin-BCM in the form of 500 times stock solutions in absolute ethanol. An average of 14-20 germs, 7-10 on RA or Ch55-supplemented BCM or transferrin-BCM, the other 7-10 on BCM or transferrin-BCM were used in each experiment. In order to avoid possible culture artifacts due to differences in the developmental stages of the molars at the time of explantation, the explants cultured with a given retinoid concentration (experimental group) all came from the same litter. Likewise, the control group, cultured in the absence of retinoids, consisted of the contralateral molars. In one experiment, day-11 mandibular arches were cultured on transferrin-BCM or on transferrin-BCM supplemented with 1.5×10^{-8} M RA. At this stage the dental lamina of the molar has not yet formed. Formation of molar rudiments was consistently observed, under the dissecting microscope, after 3 days in culture. These molars, at the early bud-stage, were dissected and returned to the same media for 9 days. At the end of the culture period, the explants were fixed, then processed for histology or immunohistochemistry.

Histological procedures

Cultured teeth were fixed in Bouin-Hollande and embedded in paraffin. Serial 5 mm sections were stained with Mallory's phosphotungstic acid – hematoxylin.

Cell proliferation assay

Day-16 molars were cultured for 4, 6 or 7 days on BCM (control groups) or on BCM supplemented with 1.5×10^{-7} M RA (experimental groups) then immersed into the same liquid media containing 5-bromo-2'-deoxyuridine (BrdU, included in Amersham's labeling reagent, diluted 1:1000) and cultured again for 20 h. The molars were then washed in Hanks balanced salt solution (3x10 min), fixed in Bouin-Hollande (5 h; 24°C), washed with distilled water (3x10 min) then 70% ethanol (16 h), dehydrated through graded ethanol series, cleared and embedded in paraffin. BrdU incorporated into DNA was located on 5 μm serial sections with a specific mouse monoclonal antibody and immunoperoxidase labeling, following the manufacturer's instructions (Amersham). The immunostained sections were counterstained with 0.01% Safranin O (C.I. 50240) for 5 min, dehydrated and mounted in Eukitt (Labonord, France).

Enzymatic removal of the ECM and immunofluorescence detection of laminin

Day-14 and day-16 molars were cultured for 4 days on BCM (day-16 explants) or BCM-transferrin with or without 1.5×10^{-7} M RA (day-14 explants), then either immediately fixed for immunohistochemistry (see below) or treated with trypsin and cultured again. In the latter case, the cultured molars were incubated in 1% trypsin (Difco, 1:250) in Hanks balanced salt solution until partial dissociation of the enamel organ and dental papilla (approximately 1 h at 4°C; Ruch *et al.*, 1976). This results in removal of the basement membrane and of the dental papilla ECM (Meyer *et al.*, 1978; Lesot *et al.*, 1981; Osman and Ruch, 1981; Mark *et al.*, 1990). The trypsinized explants were cultured again for 30 h, 2 days or 4 days.

For immunohistochemistry, the explants were fixed in freshly prepared, ice cold 4% paraformaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.2) for 6 h at 4°C, washed in PBS (consisting of 0.12 M NaCl, 10.40 mM $Na_2H\,PO_4$ and 3.16 mM $KH_2\,PO_4$, pH 7.2; 3 x 30 min; 4°C), then soaked in 20% sucrose in PBS (16 h; 4°C), embedded in Tissue Teck OCT (Miles Scientific) and quickly frozen in the vapor phase of liquid nitrogen. Serial sections, 8 mm thick, were cut with a cryostat and picked up on polylysine-coated glass slides.

Following rehydration, the sections were incubated with monospecific, affinity-purified rabbit anti-mouse antibodies to Engelbreth Holm Swarm (EHS) laminin (diluted 1/200; Simo *et al.*, 1991). Areas of the histological sections which reacted with these antibodies were subsequently visualized by indirect immunofluorescence employing fluorescein-conjugated goat

antibodies to rabbit IgG (diluted 1/40; Jackson Immunoresearch). The immunostained sections were mounted in buffered glycerol containing paraphenylenediamine.

For controls, non-immune rabbit serum (diluted 1:50) replaced the anti-laminin antibodies in the immunostaining sequence.

Acknowledgments

We thank Drs. Y. Hashimoto and K. Shudo for their generous gift of Ch55, and Dr. Simon-Assmann for providing the anti-laminin antibody, Mr. A. Ackermann for technical assistance and Mr. B. Boulay for photographic processing. Supported by a grant INSERM CJF 88-08 and by the Fondation pour la Recherche Médicale.

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Accepted for publication: October 1992