Int. J. Dev. Biol. 39: 383-393 (1995)

Original Article

Polyamine depletion-mediated effects on murine odontogenesis are dependent on tooth developmental stage and culture conditions

AMEL GRITLI-LINDE[1]*, JEAN-VICTOR RUCH[2] and ANDERS LINDE[1]

[1]*Department of Oral Biochemistry, Faculty of Odontology, Göteborg University, Sweden and*
[2]*Institut de Biologie Médicale, Faculté de Médecine, Université Louis Pasteur, Strasbourg, France*

ABSTRACT Polyamines are known to play a central role in processes such as growth and development. Virtually nothing is known about their importance in tooth development, an attractive and frequently used experimental model for studies of developmental processes. A polyamine-depleted state was created in tooth cells in an organ culture system. First lower molar germs from 16- and 17.5-day old mouse fetuses were used. α-difluoromethylornithine (DFMO) and methylglyoxal bis-(guanylhydrazone) (MGBG) were used to deplete the cells from their polyamine content. Polyamine interconversion and catabolism were prevented by aminoguanidine sulfate (AG). In day-16 germs cultured in serum-containing medium, DFMO reduced the frequency of cycling cells as shown by [^{3}H]thymidine incorporation, and induced a delay of odontoblast differentiation of about 24 h. Under the same conditions, MGBG induced an arrest of histo-morphogenesis, correlated to a significant decrease in the rate of cell proliferation. Addition of polyamines prevented DFMO- and MGBG-induced delay of tooth differentiation. Interestingly, MGBG did not delay the terminal differentiation of odontoblasts and ameloblasts in cultured day-17.5 molars; in these, cells at the tip of the cusps are only a few hours before their withdrawal from the cell cycle. In serum-deprived medium, dental cytodifferentiations did not occur. Addition of putrescine or spermidine to serum-free media, however, allowed for tooth morphogenesis and cytodifferentiation. Tooth explants in a serum-deprived medium reacted to DFMO in a cytocidal fashion, whereas MGBG showed only a mild toxicity in some cell types. Addition of putrescine to DFMO-containing medium prevented its cytotoxic effect. Addition of spermidine to MGBG-containing medium not only prevented its mild toxicity but also allowed for predentin secretion by differentiated odontoblasts. The results are discussed with regard to the well-established developmental events of tooth germs cultured *in vitro* and with respect to present knowledge of polyamine metabolism and their involvement in cellular processes.

KEY WORDS: *odontogenesis, ornithine decarboxylase, S-adenosylmethionine decarboxylase, α-difluoromethylornithine, methylglyoxal bis-(guanylhydrazone)*

Introduction

The polyamines spermine, spermidine, and putrescine are involved in cellular growth and differentiation (Heby, 1981; Villanueva, 1983; Tabor and Tabor, 1984; Pegg, 1986). However, their exact mechanisms of action at the molecular level are yet to be fully elucidated. Tooth development would constitute a relevant experimental system for such studies, but no evidence has been provided so far to demonstrate a role of polyamines in tooth morphogenesis and differentiation.

The importance of polyamines in cellular processes has been demonstrated in studies using polyamine antimetabolites. The most used inhibitors are α-difluoromethylornithine (DFMO) and methylglyoxal bis-(guanylhydrazone) (MGBG). DFMO is a highly selective, enzyme-activated, irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway (Metcalf *et al.*, 1978). DFMO causes a rapid decrease of the cellular putrescine and spermidine content (Mamont *et al.*, 1978; Oredsson *et al.*, 1980; Heby and Jänne, 1981). MGBG, an antileukemic drug, is a potent competitive inhibitor of S-adenosylmethionine decarboxylase (AdoMetDC) (Williams-Ashman and Schenone, 1972). The cellular polyamine profile

Abbreviations used in this paper: AG, aminoguanidine sulfate; DFMO, α-difluoromethylornithine; HBSS, Hanks' balanced salt solution; MGBG, methylglyoxal bis-(guanylhydrazone); ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; SCM, serum-containing medium; SFM, serum-free medium; ^{3}H-TdR, [^{3}H]thymidine.

***Address for reprints:** Department of Oral Biochemistry, Medicinaregatan 7B, S-413 90 Göteborg, Sweden. FAX: 46-31-418122.

0214-6282/95/$03.00

Printed in Spain

TABLE 1

DENTAL CYTODIFFERENTIATION AFTER POLYAMINE DEPLETION AND REPLENISHMENT COMPARED TO THE CONTROL GROUP

Culture conditions	Days in culture			
	4	5	6	7
16d+SCM (control)	PoO+PA	FO+PD+PA	FO+P D+PoA	FO+P D+A
16d+SCM+20 mM DFMO	PO+PA	PoO+PA		FO+PD±A
16d+SCM+20 mM DFMO+200 μM AG	PO+PA	PoO+PA	FO+PD+PA	FO+PD±A
16d+SCM+20 mM DFMO+200 μM AG+100 μM PT	PoO+PA	FO+PD+PA	FO+P D+PoA	
16d+SCM+20 mM DFMO+200 μM AG+20 μM SD	PoO+PA	FO+PD+PA	FO+P D+PoA	
16d+SCM+20 mM DFMO+200 μM AG+10 μM SP	PoO+PA	FO+PD+PA	FO+P D+PoA	
16d+SCM+200 μM AG	PoO+PA	FO+PD+PA	FO+P D+PoA	
16d+SCM+100 μM MGBG	T	T		
16d+SCM+10 μM MGBG	PO+PA	PO+PA	PO+PA	PO+PA
16d+SCM+10 μM MGBG+100 μM PT			FO+P D+PoA	
16d+SCM+10 μM MGBG+20 μM SD			FO+P D+PoA	
16d+SCM+10 μM MGBG+10 μM SP			FO+P D+PoA	
17.5d+SCM	FO+PD+PA			FO+ P D+A
17.5d+SCM+10 μM MGBG	FO+PD+PA			FO+ P D+A
16d+SFM (control)		PO+PA	PO+PA	
16d+SFM+20 mM DFMO		T		
16d+SFM+20 mM DFMO+200 μM PT		PO+PA		
16d+SFM+10 μM MGBG		PO+PA	PO+PA	
16d+SFM+10 μM MGBG+80 μM SD		FO+PD+PA	FO+P D+PoA	
16d+SFM+400 μM PT			FO+P D+PoA	
16d+SFM+80 μM SD			FO+P D+PoA	

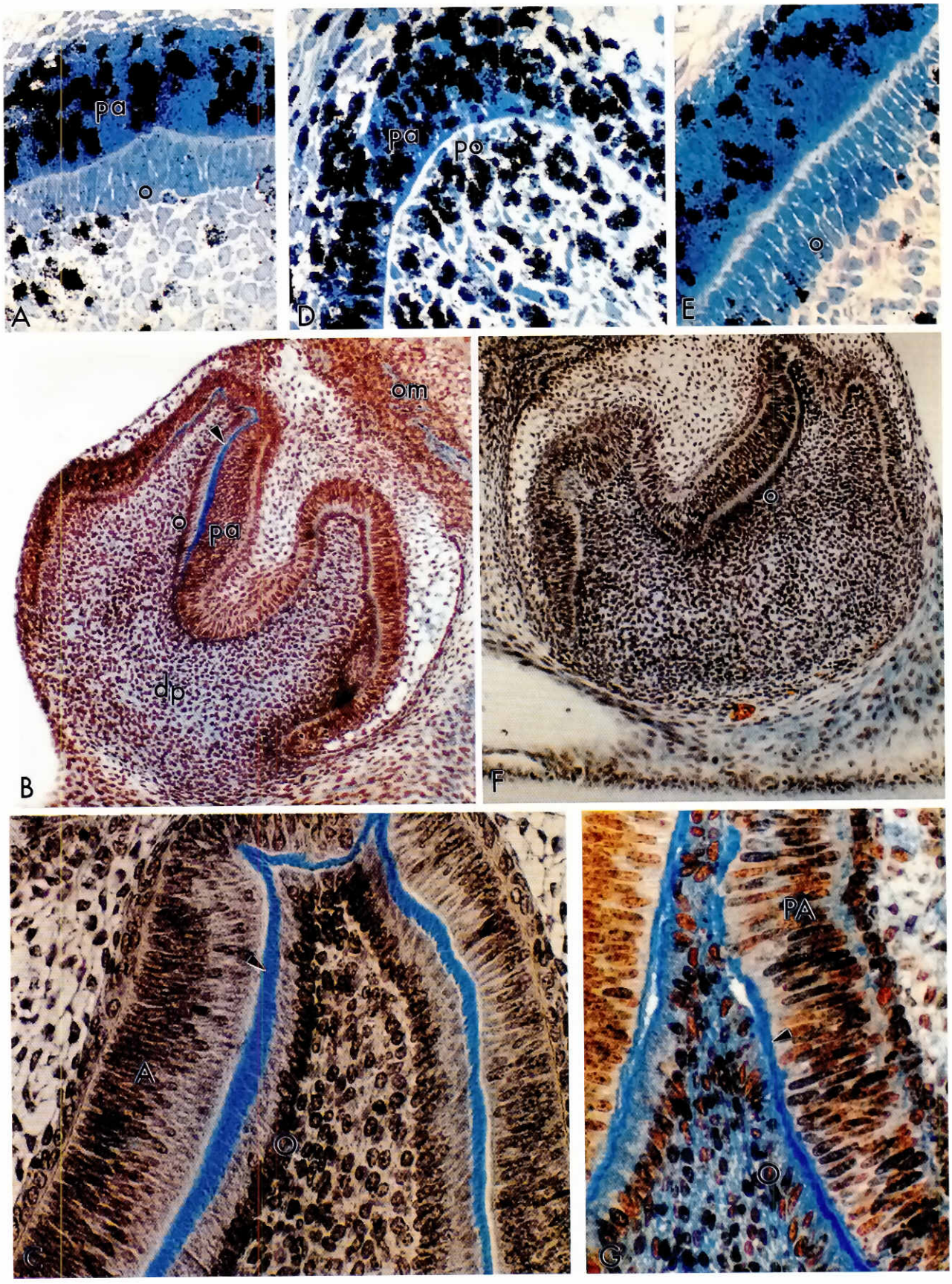
16d, day-16 tooth germs; 17.5d, day-17.5 tooth germs; SCM, serum-containing medium; SFM, serum free medium; PT, putrescine; SD, spermidine; SP, spermine; PoO, polarizing odontoblasts; PO, preodontoblasts; FO, functional odontoblasts; PA, preameloblasts; PoA, polarizing ameloblasts; A, ameloblasts; PD, thin layer of predentin; P D , thick layer of predentin; T, toxicity (dead germs).

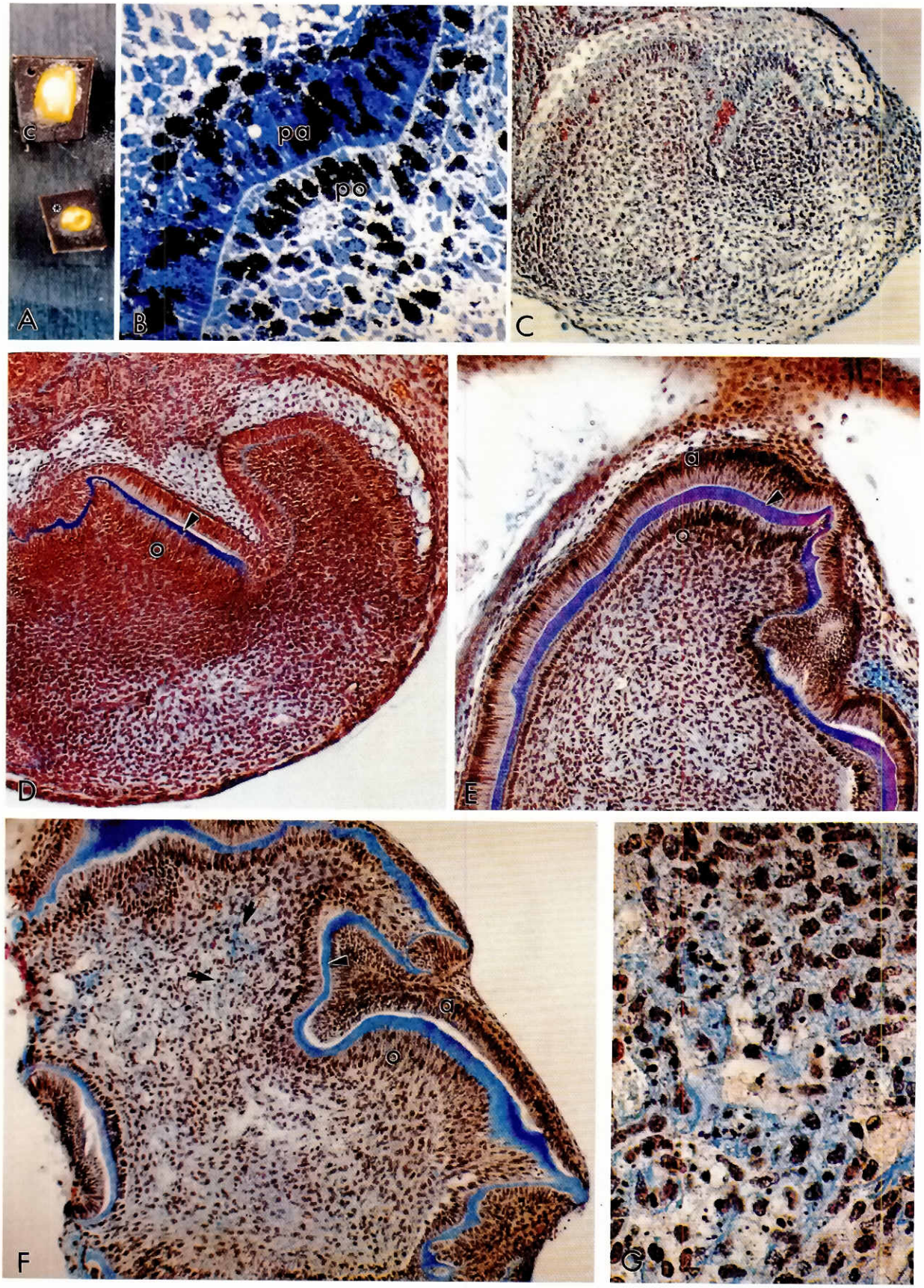
generated by MGBG differs from that produced by DFMO. Thus, MGBG effectively blocks the formation of spermidine and spermine in various cells *in vitro*, while the putrescine content is strikingly increased (Heby and Jänne, 1981).

A continuous sequence of reciprocal interactions between the epithelium originating from the first branchial arch, and specific neural crest-derived cells of the ectomesenchyme is a prerequisite for the development of an embryonic tooth (Lumsden, 1985). These interactions are probably mediated and regulated by cell-to-cell contacts, stage-specific extracellular matrices, growth factors, and DNA-binding transcription factors (Ruch *et al.*, 1983; Ruch, 1984; Partanen and Thesleff, 1989; Cam *et al.*, 1990; Thesleff *et al.*, 1990; Jowett *et al.*, 1993). By such mechanisms, the progressive development of the tooth primordium (the dental lamina, and the bud, cap, and bell stages) is brought about, and the overt differentiation of odontoblasts, secreting predentin/dentin, and of ameloblasts, secreting enamel matrix, takes place. The terminal differentiation of odontoblasts and ameloblasts occurs according to a cusp-specific, temporo-spatial pattern. In the murine first lower molar, the terminal differentiation of odontoblasts *in vivo* is initiated in the cuspal tip on day 18 and progresses radially to the cervical loop. The first post-mitotic ameloblasts appear 16-24 h after the odontoblasts have become functional (Ruch, 1990). *In vitro*, the same events occur with regard to morphogenesis and cytodifferentiation, but with a delay in time (Ruch *et al.*, 1976). In addition to being a frequently used model for the investigation of biomineralization (Linde and Goldberg, 1993), odontogenesis thus constitutes an advantageous experimental system to study developmental processes. In spite of this, little has been done in terms of studies of the role of polyamines during odontogenesis. The only data presently available is the demonstration of ODC activity in teeth at different developmental stages (Gritli-Linde and Linde, 1994) and the localization of AdoMetDC in murine tissues, including teeth, by immunohistochemical techniques (Gritli-Linde *et al.*, 1995).

The aim of the present investigation was to study the role of polyamines in tooth development by creating a polyamine-depleted state in tooth cells in organ culture. DFMO and MGBG were used to inhibit ODC and AdoMetDC, respectively. Prevention of polyamine interconversion and degradation was made by aminoguanidine sulfate (AG), which inhibits both the enzyme polyamine oxidase in the alternative route to spermidine and putrescine as well as Cu^{2+}-containing amine oxidases (Seiler *et al.*, 1983).

Fig. 1. Day-16 molars grown in serum-containing medium (SCM). *Tooth explants cultured in the SCM (control)* **(A-C)**. *Autoradiograph of an explant cultured for 4 days and pulse labeled with ^{3}H-TdR, showing post-mitotic odontoblasts that did not incorporate ^{3}H-TdR. x560* **(A)**. *Molar germ cultured for 5 days showing advanced cusp morphogenesis and predentin secretion by functional odontoblasts. x140* **(B)**. *After 7 days a thick layer of predentin was secreted and ameloblasts were polarized. x560* **(C)**. *Withdrawal of odontoblasts from the cell cycle, as shown by ^{3}H-TdR incorporation, did not occur in explants cultured in the SCM+DFMO+AG for 4 days. x560* **(D)**. *Putrescine addition to the SCM containing DFMO and AG allowed for odontoblast withdrawal from the cell cycle after 4 days. x560* **(E)**. *In the presence of SCM+DFMO+AG, odontoblast polarization occurred after 5 days in culture, demonstrating a delay of about 24 h. x140* **(F)**. *After 7 days of culture in SCM+DFMO+AG, only a thin layer of predentin was secreted, and ameloblasts were essentially not differentiated. x560* **(G)**. *pa, preameloblasts; A, ameloblasts; po, preodontoblasts; o, odontoblasts; dp, dental papilla; arrowheads, predentin; om, oral mucosa.*


pa
o
A
pa
po
D
o
E
om
o
pa
dp
B
o
F
A
O
C
PA
O
G


c
A
pa
po
B
C
o
D
a
o
E
a
o
F
G

Results

The results regarding culture conditions and days in culture are summarized in Table 1. In each experimental group, 5 to 7 tooth explants were used. Each experiment was performed at least twice with highly reproducible results. The photomicrographs shown were taken from the region of the tooth where histomorphogenesis was the most advanced.

At the onset of the culture, day-16 first lower molars are at the bell stage and contain replicating preodontoblasts and preameloblasts. In these, cusp morphogenesis has just begun. In day-17.5 first lower molars, preodontoblasts at the tip of the principal cusp, are engaged in their last cell cycle and will overtly differentiate on day 18.

Day-16 molars grown in SCM

Tooth explants cultured for 4 days in SCM without adding any drug, displayed polarized odontoblasts at the tip of the principal cusp. These cells did not incorporate ^{3}H-TdR, thus demonstrating a withdrawal from the cell cycle (Fig. 1A). After 5 days in culture, predentin was secreted by functional odontoblasts (Fig. 1B). After 6 days, a thicker layer of predentin had been secreted, and ameloblasts were polarizing. Ameloblast polarization occurred after 7 days of culture (Fig. 1C).

Day-16 molars treated with DFMO in SCM and prevention study

Tooth germs cultured in the presence of DFMO alone or in combination with AG, demonstrated a delay of odontoblast and ameloblast differentiation as compared to control molars. After 4 days of culture with DFMO alone or in combination with AG, odontoblast polarization did not occur and the cells were still incorporating ^{3}H-TdR (Fig. 1D). After 5 days of culture, molars treated with DFMO alone or in combination with AG, showed polarizing odontoblasts at the tip of the principal cusp (Fig. 1F). Tooth explants cultured for 6 days in the presence of DFMO and AG, demonstrated secretion of a thin layer of predentin. No ameloblast polarization occurred in the presence of DFMO alone or with AG during 7 days of culture (Fig. 1G).

Tooth explants cultured in the presence of DFMO plus AG and the different polyamines (prevention study), prevented the DFMO-induced delay of dental cytodifferentiation. For example, germs cultured with DFMO plus AG and putrescine (or spermidine, or spermine) for 4 days displayed polarizing odontoblasts at the tip of the principal cusp (Fig. 1E).

Day-16 molars treated with AG in SCM

Tooth germs cultured in the presence of AG alone showed normal cusp morphogenesis and dental cytodifferentiation. Odontoblast differentiation was evident after 4 days of culture. Predentin secretion occurred during the 5th day, and polarizing ameloblasts were seen after 6 days (not shown).

Day-16 molars treated with MGBG in SCM and prevention study

MGBG at a 100 μM concentration was toxic for tooth explants and the surrounding mucosa (not shown). Tooth explants grown in the presence of 10 μM MGBG were considerably smaller than molars cultured in the control medium (Fig. 2A). This is illustrated by the fact that fewer sections ($p<0.01$; Student's t test) could be taken by serial sectioning. While 41.7±5.9 sections could be obtained from the controls, only 22.0±5.7 sections were possible to harvest from germs cultured in the presence of MGBG (mean values±S.D.; n= 6). Furthermore, the oral mucosa was atrophic. In the presence of the drug, cuspal morphogenesis did not progress and dental cytodifferentiation did not occur. The germs were blocked at the early bell stage during 4, 5 (Fig. 2B), 6 (Fig. 2C), and 7 days of culture with a mild cytotoxicity in cells of the dental papilla, revealed as a small number of cells with pycnotic nuclei.

The effects of MGBG on tooth morphogenesis and differentiation were prevented by polyamine addition to the culture medium containing the drug. Tooth explants, cultured in SCM+10 μM MGBG+20 μM spermidine (or 10 μM spermine) (prevention study), displayed normal morphogenesis and cytodifferentiation events. During 6 days in culture, the teeth demonstrated predentin secretion by functional odontoblasts, and ameloblasts were polarizing (Fig. 2D). Surprisingly, predentin was also secreted in the molars grown in the presence of MGBG and 100 μM putrescine (not shown).

Day-17.5 molars grown in SCM

Both control molar explants and explants cultured in the presence of MGBG showed normal development and similar sizes. After 4 days, cusp morphogenesis was advanced and odontoblasts had secreted predentin. After 7 days in culture, polarization of ameloblasts was evident (Fig. 2E,F). Furthermore, in the presence of MGBG no disturbance of the cytodifferentiation gradient was detected, but some cytotoxicity signs were observed in some cells of the dental papilla (Fig. 2F,G).

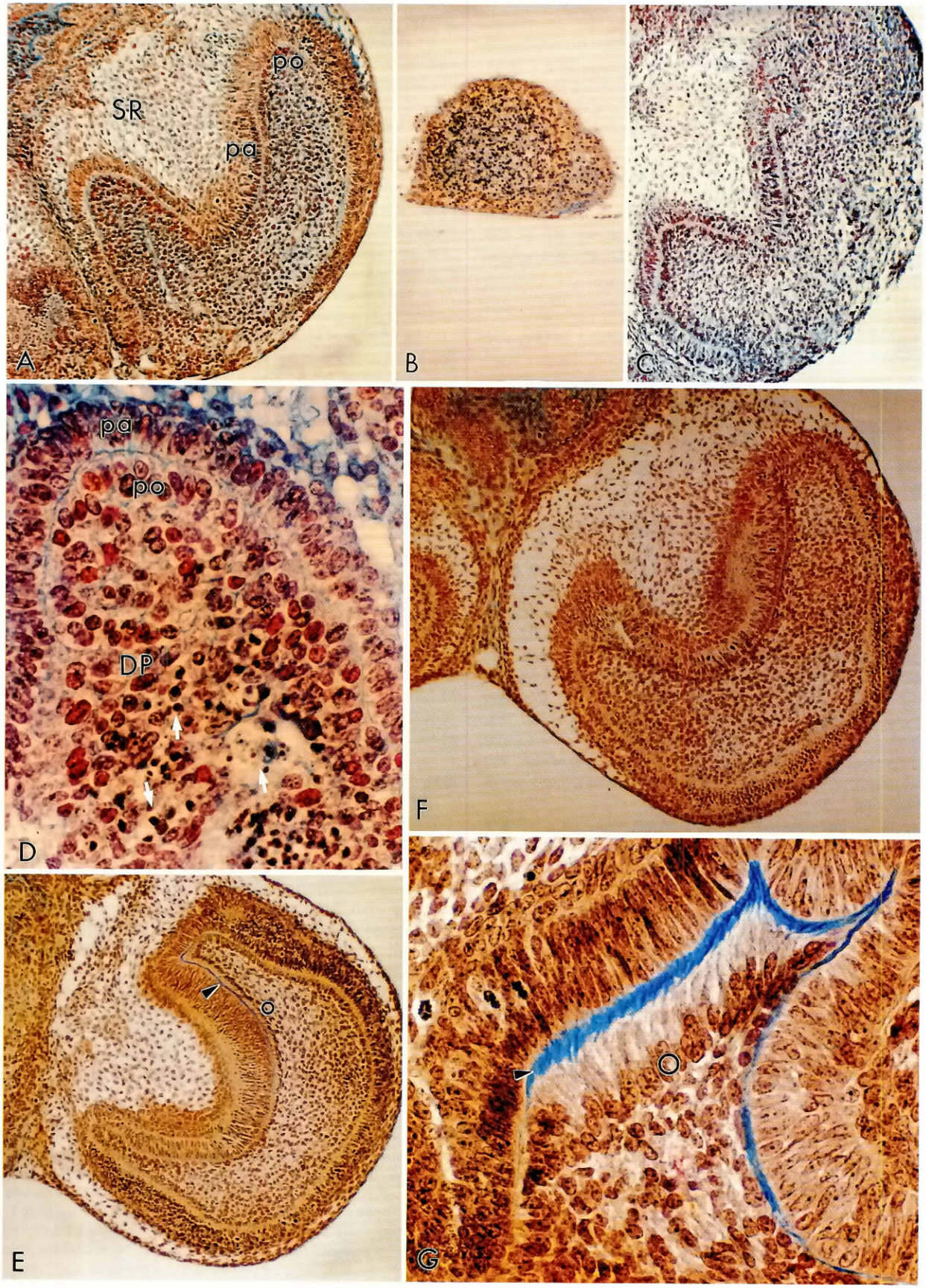
Day-16 molars grown in SFM

Serum deprivation induced a delay of odontoblast differentiation. Under the same culture conditions, no odontoblast differentiation was evident after 5 and 6 days (Fig. 3A,F).

DFMO at a 20 mM concentration displayed a cytocidal effect on all the cell lineages of the tooth as well as on the surrounding mucosa after 5 days of culture (Fig. 3B). Addition of 200 μM putrescine to the medium containing DFMO partially prevented the cytotoxic effects of the drug but did not allow for odontoblast polarization (Fig. 3C).

Explants cultured in the presence of 10 μM MGBG for 5 or 6 days displayed some cytotoxic effects only in some cells of the dental papilla; preameloblasts and preodontoblasts were normal. Furthermore, the molars were still at the early bell stage (Fig. 3D). Surprisingly, addition of 80 μM spermidine to the medium contain-

Fig. 2. Molars cultured in serum-containing medium (SCM). *Day-16 explants cultured for 6 days in SCM+10 μM MGBG (asterisk) were considerably smaller than explants cultured in the SCM alone (c). Specimens immersed in Bouin's fixative for a few seconds. x9* **(A)**. *Day-16 tooth explant pulse-labeled with ^{3}H-TdR, grown for a total period of 5 days in SCM+MGBG. x560* **(B)**. *Day-16 molar germ cultured for 6 days in SCM+10 μM MGBG. x140* **(C)**. *Day-16 molar germ grown for 6 days in SCM+10 μM MGBG+20 μM spermidine, showing predentin secretion by functional odontoblasts as well as polarizing ameloblasts. x140* **(D)**. *Day-17.5 molar cultured for 7 days in SCM. x140* **(E)**. *Day-17.5 molar cultured in SCM+10 μM MGBG for 7 days, showing normal development except for a mild toxicity in the dental papilla (arrows). x140* **(F)**. *High magnification of dental papilla from day-17.5 molar cultured in SCM+10 μM MGBG, showing a mild toxicity in some cells. x560* **(G)**. *pa, preameloblasts; po, preodontoblasts; a, ameloblasts; o, odontoblasts; arrowheads, predentin.*


po
SR
pa
A
B
C
pa
po
DP
D
F
o
E
O
G

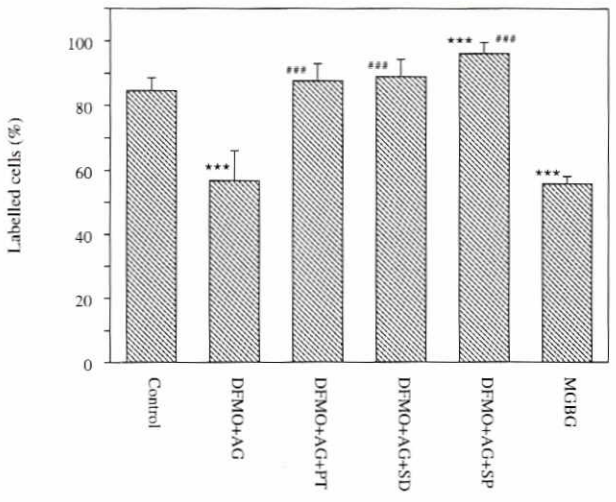


Fig. 4. [^{3}H]thymidine incorporation in tooth explants. *Frequency of cycling cells (expressed as % of total number of cells) in day-16 tooth germs cultured for a total period of 5 days in the standard medium alone (control), in the presence of inhibitors, or with both inhibitors and polyamines added. PT, putrescine; SD, spermidine; SP, spermine. "***" denotes a statistically significant difference (p<0.0002) from the percentage of cycling cells in tooth germs cultured in standard medium. "###" denotes a statistically significant difference (p<0.0001) from the percentage of cycling cells in tooth germs cultured in the presence of DFMO+AG.*

ing MGBG not only prevented cytotoxicity but also allowed for normal cusp morphogenesis and predentin secretion by odontoblasts during 5 (Fig. 3E) and 6 days of culture.

Addition of 400 μM putrescine or 80 μM spermidine to the control SFM allowed for predentin secretion by odontoblasts during 6 days of culture (Fig. 3G).

^{3}H-TdR incorporation

The frequency of labeled cells (expressed as % of total number of cells) in day-16 tooth germs cultured for a total period of 4 days is given in Fig. 4. In germs cultured in the control SCM the percentage of labeled cells was 84.6±4.0% (n= 10). The mean number of labeled cells decreased considerably (p<0.0001) in DFMO-treated germs as compared to controls. Addition of the separate polyamines caused a strongly significant (p<0.0001; n= 9) increase in the number of ^{3}H-TdR-positive cells, compared to the DFMO-treated germs. Interestingly, while there was no statistical difference between controls, on the one hand, and germs replenished with putrescine or spermidine on the other, replenishment with spermine caused an increase in the percentage of labeled cells (96.1±3.5%; p= 0.0002) compared to control germs. Upon MGBG treatment, the mean number of labeled cells (55.7±2.4) significantly (p<0.0001; n= 7) decreased as compared to control explants.

Discussion

The results obtained show that inhibition of ODC by DFMO induced a delay of odontoblast differentiation of about 24 h. Polyamine supplementation prevented this delay. On the other hand, MGBG induced an arrest of tooth development at the early bell stage or had no effect at all, depending on the developmental stage of the tooth anlagen at the onset of culture. Also the MGBG-induced arrest of tooth development was prevented by supplementation with polyamines. In the absence of serum, DFMO was very cytotoxic and MGBG only slightly toxic for tooth cells. Putrescine supplementation prevented this DFMO toxicity, whereas spermidine supplementation allowed for tooth differentiation in the presence of MGBG. Control molars grown in serum-free medium displayed a delay of odontoblast differentiation, while addition of polyamines to the medium allowed for dental cytodifferentiation.

The biosynthetic pathways of polyamines have been well characterized (for review, see Pegg, 1988; Seiler and Heby, 1988). This pathway is schematically depicted in Fig. 5.

DFMO effects in serum-containing medium

Bovine serum, including fetal calf serum, contains copper-containing amine oxidases, enzymes involved in polyamine degradation and generation of toxic compounds (Seiler and Heby, 1988; Morgan, 1989). This hampers the use of cell cultures in polyamine research, and AG was thus used in order to overcome this problem (Stjernborg and Persson, 1993). AG alone did not induce changes in tooth development, indicating that the alterations of tooth differentiation observed was indeed caused by the inhibition of ODC by DFMO. Furthermore, supplementation of the medium with polyamines did not induce toxicity within the germs, suggesting that AG was efficient against amine oxidases.

DFMO was found to induce a delay of odontoblast differentiation of about 24 h. Supplementation of the medium containing DFMO and AG with putrescine, spermidine, or spermine (or with these three polyamines together; not shown), prevented this effect of DFMO. The prevention of the DFMO effect by polyamines was not due to any competition between them, since it is well established that DFMO, in contrast to the polyamines, enters cells by passive diffusion. These results suggest that the effects mediated by DFMO were actually caused by polyamine depletion.

MGBG effects in serum-containing medium

At the onset of culture (day 16), the germ of the first lower molar is at the early bell stage, and histogenesis and cusp formation have been initiated. At this stage, this organ consists of two interacting

Fig. 3. Day-16 molar explants cultured in serum-free medium (SFM). *In tooth germs cultured for 5 days in SFM (control), odontoblast polarization was delayed. x140* **(A)**. *In SFM+20 mM DFMO, the tooth and the surrounding mucosa responded to the drug in a cytocidal fashion after 5 days. x140* **(B)**. *The cytotoxic effect of DFMO was prevented in molar explants grown for 5 days in SFM+20 mM DFMO+200 μM putrescine, but odontoblasts did not polarize. x140* **(C)**. *Molar explants cultured in SFM+10 μM MGBG displayed a mild toxicity only in some cells of the dental papilla (white arrows). Preodontoblasts, preameloblasts and cells of the stratum intermedium and the stellate reticulum were normal. Furthermore, odontoblast polarization did not occur. x560* **(D)**. *Supplementation of the MGBG-containing medium with spermidine (SFM+10 μM MGBG+80 μM spermidine) not only prevented some cytotoxic response but also allowed for predentin secretion by differentiated odontoblasts. x140* **(E)**. *Molar germ grown for 6 days in SFM (control), showing a delay of odontoblast polarization. x140* **(F)**. *Molar explant grown for 6 days in SFM+80 μM spermidine, showing normal cusp morphogenesis and predentin secretion by functional odontoblasts. x560* **(G)**. *po, preodontoblasts; pa, preameloblasts; DP, dental papilla; SR, stellate reticulum; arrowheads, predentin; O, odontoblasts.*

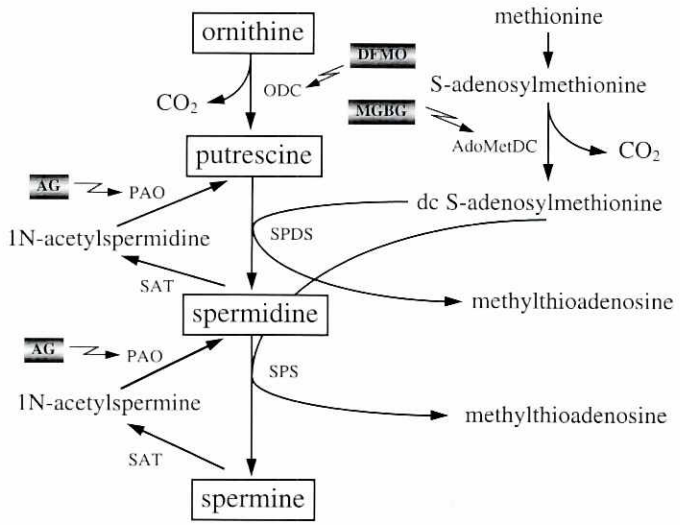


Fig. 5. Schematic display of polyamine metabolism. *Scheme modified after Seiler and Heby (1988).*

tissues containing proliferating cells: 1) the inner dental epithelium of the enamel organ, which will eventually generate ameloblasts, and 2) the dental papilla, consisting of mesenchymal cells, which will give rise to odontoblasts.

In serum-containing medium, MGBG (10 μM) induced an arrest of tooth development at the early bell stage, without causing any toxic effects in cells of the odontoblastic or ameloblastic lineages. Even though spermidine and spermine prevented the effect of MGBG, this phenomenon does not necessarily suggest that MGBG solely acted through a depletion of these two polyamines. Although MGBG is a potent inhibitor of AdoMetDC, it exerts several side effects not related to its inhibition of AdoMetDC or to a decrease in spermidine and spermine pools (Pathak *et al.*, 1977; Pegg, 1988). Furthermore, eukaryotic cells appear to have at least one polyamine transport system (Pegg, 1988; Seiler and Dezeure, 1990), and MGBG and spermidine have structural similarities to an extent that the former enters the cells by using the same cell membrane polyamine carrier (Heby, 1981; Williams-Ashman and Pegg, 1981). Prevention of MGBG effects by spermidine and spermine could thus also result from a competition between the drug, on one hand, and spermidine and spermine, on the other, for the membrane carrier.

Depending on the cell type, it has been shown that in some cells polyamines share the same single carrier (Porter *et al.*, 1984), and in others more than one pathway for polyamine uptake exists, each carrier having different affinities for a given polyamine (Byers *et al.*, 1987; Kumagai and Johnson, 1988). A competition between putrescine and MGBG for the putative polyamine carrier was probably the cause of abrogation of MGBG-mediated arrest of tooth development by putrescine. This finding constitutes thus an indirect evidence for the existence of the same transporter for polyamines in tooth cells.

It is well known that MGBG induces an accumulation of putrescine due to a blockage of its utilization in the cells. If one assumes that arrest of tooth development in serum-containing medium supplemented with the drug was partly due to depletion of the intracellular spermidine and spermine pools, it could be speculated that the two polyamines might play a more important role in tooth cell differentiation processes than putrescine.

Effects of MGBG on 17.5-day tooth explants in serum-containing medium

Inhibition of polyamine synthesis during early development completely prevents differentiation at gastrulation in different species (Emanuelsson and Heby, 1978; Löwkvist *et al.*, 1980, 1983), including mammals (Fozard *et al.*, 1980; Slotkin *et al.*, 1983). Nevertheless, when DFMO treatment is initiated at the end of gastrulation or at the onset of early organogenesis, the *in ovo* development of chick is only retarded (Löwkvist *et al.*, 1987). When applied on day-17.5 tooth germs, namely a few hours before polarization of the first odontoblasts, MGBG was found to induce neither any arrest nor a delay in development. There is presently no satisfactory explanation for this observation, unless it is assumed that a critical period exists for MGBG to be efficient. Another possibility would be that an inactivation of the polyamine carrier in the cells occurs during or a few hours before their withdrawal from the cell cycle.

Effects of polyamine depletion in serum-free medium

Control tooth germs, cultured in serum-free medium, did not differentiate during 5 and 6 days of culture. This may be due to a deficiency in important factors provided by the serum. Surprisingly, supplementation of MGBG-containing serum-free medium with high concentrations of spermidine not only prevented the mild cytotoxic effects of the drug, but also allowed for normal morphogenesis and predentin secretion by odontoblasts. Furthermore, addition of high concentrations of putrescine and spermidine separately to the serum-free medium (without MGBG) allowed for normal morphogenesis and cytodifferentiation. Together, these findings suggest that at least some effects of MGBG were due to polyamine depletion, and that polyamines are important for tooth cell differentiation.

Unlike tooth explants cultured in serum-containing medium in which DFMO induced only a delay of cytodifferentiation, tooth cells and cells of the overlying mucosa responded to the drug in a cytocidal fashion in serum-free medium. Supplementation with putrescine counterbalanced DFMO-induced cell death. Knowing that, among other important roles, polyamines are necessary for protein synthesis, these findings suggest that DFMO-induced cell death in serum-deprived medium was related to inhibition of synthesis of proteins of vital importance for the cells, as a result of polyamine depletion.

Possible mechanisms for polyamine depletion-induced effects on tooth development

It is well known that cell growth and division are required to give rise to a certain tissue mass before informational gradients can be generated to regulate differentiation (Wolpert, 1978). As for the tooth, experimental data suggest that the genetic programme of odontoblasts and ameloblasts might determine a minimal number of cell cycles before these cells are able to respond to specific epigenetic signals, triggering their terminal differentiation (Ruch *et al.*, 1983; Ahmad and Ruch, 1987).

Polyamine synthesis inhibitors are known to reduce the growth rate of cells by altering the cell cycle in normal and tumor cells (Rupniak and Paul, 1978; Sunkara *et al.*, 1979; Oredsson *et al.*, 1986; Löwkvist *et al.*, 1987; Huang *et al.*, 1994). In this study, DFMO induced a delay in odontoblast differentiation. Furthermore, the number of cycling cells decreased in the presence of the drug. From the foregoing account, these results might be attributed to a general slowing-down of the cell cycle traverse, or to a lengthening

of the G1 phase by DFMO, leading to a delay in odontoblast and ameloblast withdrawal from the cell cycle.

MGBG-induced arrest of tooth development was expressed by a decrease in size and an inhibition of cytodifferentiation of tooth germs even after 7 days in culture. The number of cycling cells also decreased. These MGBG-mediated effects may be attributed to an impairment of cell proliferation, probably as a result of a blocking of the cell cycle traverse. DNA flow-cytometric analysis is necessary to determine the exact effect of DFMO and MGBG on cell cycle progression of dental cells.

Polyamines have been involved in the complex process of bone and cartilage differentiation (Rath and Reddi, 1981; Takano *et al.*, 1981). We have previously shown that active ODC expression was higher in differentiated tooth cells than in not yet differentiated ones (Gritli-Linde and Linde, 1994). Furthermore, immunolocalization of AdoMetDC protein in developing teeth displayed the same distribution pattern as ODC (Gritli-Linde *et al.*, 1995). Considering these observations, and in view of the results of the present study, it is suggested that polyamines play an important role in tooth development, not only within the machinery of cell proliferation, but also in differentiation processes. Furthermore, the tooth development model offers a good tool in which to investigate the mechanisms of action of polyamines.

Materials and Methods

Chemicals

DFMO and AG were generously provided by the Marion Merrell DOW Research Institute (Strasbourg, France). A 1 M stock solution of DFMO was prepared in Hanks' balanced salt solution (HBSS), pH adjusted to neutrality, and stored at -20°C. Putrescine, spermidine, spermine, MGBG and ascorbic acid were from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, kanamycin, and glutamine were from Gibco BRL Life Technologies (Gaithersburg, MD, USA). [^{3}H]thymidine (specific activity 25 Ci/mmol) was from Amersham (Little Chalfont, Bucks., UK).

Organ cultures

Mandibular first molars were dissected out from 16- and 17.5-day old, post-conception NMRI mouse fetuses (vaginal plug= day 0) and cultured on Millipore filters (0.45 μm pore size) in an organ culture system. For control studies the germs were grown in a serum-containing medium (SCM) consisting of RPMI 1640, supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 μg/ml kanamycin and 180 μg/ml ascorbic acid. In order to determine the effects of polyamine depletion on tooth development, molar germs were cultured in the SCM supplemented with different inhibitors of polyamine synthesis. As for prevention studies, the germs were cultured in the SCM supplemented with the same inhibitors and the three different polyamines. The polyamines were added individually to the medium containing the inhibitors at the onset of the culture. Day-16 molars were also grown in a serum-deprived medium (SFM) in the presence or absence of inhibitors and/or polyamines. The germs were grown for varying time periods between 4 and 7 days at 37°C in the presence of 5% CO_2 in air. The culture media were changed every second day. Tables 2 and 3 summarize the different culture conditions.

Histology

Tooth germs were fixed in Bouin's fixative overnight and processed for paraffin embedding. Serial 5 μm section were stained with Ladewig's hematoxylin.

Evaluation of the volume of tooth germs

To determine the effects of MGBG on the size of the teeth, the number of frontal serial sections (5 μm) including dental tissues was determined from the germs cultured for 6 days in the SCM (control) and SCM+MGBG.

TABLE 2

DAY-16 (16d) AND DAY-17.5 (17.5d) MOLARS CULTURED IN SERUM-CONTAINING MEDIUM (SCM)

Culture conditions	Days in culture			
	4	5	6	7
16d+SCM (control)	+	+	+	+
16d+SCM+20 mM DFMO	+	+		+
16d+SCM+20 mM DFMO+200 μM AG	+	+	+	+
16d+SCM+20 mM DFMO+200 μM AG+100 μM PT	+	+	+	
16d+SCM+20 mM DFMO+200 μM AG+20 μM SD	+	+	+	
16d+SCM+20 mM DFMO+200 μM AG+10 μM SP	+	+	+	
16d+SCM+200 μM AG	+	+	+	
16d+SCM+100 μM MGBG	+	+		
16d+SCM+10 μM MGBG	+	+	+	+
16d+SCM+10 μM MGBG+100 μM PT			+	
16d+SCM+10 μM MGBG+20 μM SD			+	
16d+SCM+10 μM MGBG+10 μM SD			+	
17.5d+SCM (control)	+			+
17.5d+SCM+10 μM MGBG	+			+

AG, aminoguanidine sulfate; DFMO, α-difluoromethyl-ornithine; MGBG, methylglyoxal bis-(guanylhydrazone); PT, putrescine; SD, spermidine; SP, spermine.

[^{3}H]thymidine incorporation and autoradiography

Day-16 molars cultured for a total period of 4 or 5 days in the SCM (control), SCM+20 mM DFMO+200 μM AG, SCM+20 mM DFMO+200 μM AG+100 μM putrescine (or 20 μM spermidine, or 10 μM spermine), and SCM+10 μM MGBG, respectively, were pulse-labeled for the last 24 h with 2 μCi/ml [^{3}H]thymidine (^{3}H-TdR). The mean duration of the cell cycle in both epithelial and mesenchymal dental cell lineages is about 20 h (Ahmad and Ruch, 1987; Mark *et al.*, 1992). A 24 h ^{3}H-TdR pulse-labeling allows for the distinction between cycling cells from those that are already post-mitotic when ^{3}H-TdR is added to the culture medium. After labeling, the molars were washed with HBSS 3 times for 20 min and fixed overnight at 4°C in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. The explants were then rinsed in the buffer and post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer for 30 min. After dehydration in graded alcohols, the specimens were embedded in Agar 100 epoxy resin (Agar, Stansted, Essex, UK). For autoradiography, semi-thin serial sections were coated with Ilford K5 nuclear emulsion and stored at 4°C. After 7 days of exposure, the autoradiographic preparations were developed in Kodak D 19, fixed in 15% $Na_2S_2O_3$ and stained with Richardson's azure II-methylene blue.

The percentages of labeled nuclei counted over some 1000 cells were established by counting ^{3}H-TdR-positive cells in non-adjacent semi-thin

TABLE 3

DAY-16 MOLARS CULTURED IN SERUM FREE MEDIUM (SFM)

Culture conditions	Days in culture	
	5	6
SFM (control)	+	+
SFM+20 mM DFMO	+	
SFM+20 mM DFMO+200 μM PT	+	
SFM+10 μM MGBG	+	+
SFM+10 μM MGBG+80 μM SD	+	+
SFM+400 μM PT		+
SFM+80μM SD		+

DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis-(guanylhydrazone); PT, putrescine; SD, spermidine.

sections from molars cultured for a total period of 4 days as above. Background labeling was negligible since silver grains outside the cells were absent. ^{3}H-TdR-positive cells were considered to be those containing more than six silver grains or an aggregate of grains in their nuclei. Cell counting under an oil-immersion lens was carried out using a micrometer square of 10.000 μm^2 surface area. Counting was made over the micrometer square in an area located between the top of the cusp and the cervical loop, including preameloblasts, preodontoblasts and some cells of the dental papilla. A total of 7 -10 sections were counted from each experimental group and the values were expressed as labeled nuclei in percent of total number of nuclei. Statistical analysis was performed by an unpaired t-test.

Acknowledgments

The authors gratefully acknowledge the invaluable advice of Dr. Niclaus Seiler, Dr. Shakir Sarhan and Dr. Lo Persson. Prof. Lars E. Ericsson and Dr. H-A. Hansson at the Department of Anatomy and Cell Biology, Göteborg University kindly placed equipment at our disposal. This investigation was supported by the Swedish Institute, the Faculty of Odontology, Göteborg University, the Wilhelm & Martina Lundgren Scientific Foundation, and by the Swedish Medical Research Council (grant 2789).

References

AHMAD, S. and RUCH, J.V. (1987). Comparison of growth and cell proliferation kinetics during mouse molar odontogenesis *in vivo* and *in vitro*. *Cell Tissue Kinet. 20:* 319-329.

BYERS, T.L., KAMEJI, R., RANNELS, D.E. and PEGG A.E. (1987). Multiple pathways for uptake of paraquat, methylglyoxal bis-(guanylhydrazone) and polyamines. *Am. J. Physiol. 252:* C663-C669.

CAM, Y., NEUMANN, M.R. and RUCH, J.V. (1990). Immunolocalization of transforming growth factor β1 and epidermal growth factor receptor epitopes in mouse incisors and molars with a demonstration of *in vitro* production of transforming activity. *Arch. Oral Biol. 35:* 813-822.

EMANUELSSON, H. and HEBY, O. (1978). Inhibition of putrescine synthesis blocks development of polychete *Ophryotrocha labronica* at gastrulation. *Proc. Natl. Acad. Sci. USA 75:* 1039-1042.

FOZARD, J.R., PART, M-L., PRAKASH, N.J. and GROVE, J. (1980). Inhibition of murine embryonic development by α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. *Eur. J. Pharmacol. 65:* 379-391.

GRITLI-LINDE, A. and LINDE, A. (1994). Localization of ornithine decarboxylase in mouse teeth. An *in vitro* and *in vivo* study. *Int. J. Dev. Biol. 38:* 107-115.

GRITLI-LINDE, A., HOLM, I. and LINDE, A. (1995). Localization of S-adenosylmethionine decarboxylase in murine tissues by immunohistochemistry. *Eur. J. Oral Sci. 103:* 149-156.

HEBY, O. (1981). Role of polyamines in the control of cell proliferation and differentiation. *Differentiation 19:* 1-20.

HEBY, O. and JÄNNE, J. (1981). Polyamine antimetabolites: biochemistry, specificity and biological effects of inhibitors of polyamine synthesis. In *Polyamines in Biology and Medicine* (Eds. D.R. Morris and L.J. Marton). Dekker, New York, pp. 243-310.

HUANG, H., LINARES, P.N., ADOLPHE, M. and VILLANUEVA, V.R. (1994). Simultaneous inhibition of spermidine and spermine and cell division. *Biogenic Amines 10:* 259-271.

JOWETT, A.K., VAINIO, S., FERGUSON, M.W.J., SHARPE, P. and THESLEFF, I. (1993). Epithelial-mesenchymal interactions are required for msx 1 and msx 2 gene expression in the developing murine molar tooth. *Development 117:* 461-470.

KUMAGAI, J. and JOHNSON, L.R. (1988). Characteristics of putrescine uptake in isolated rat enterocytes. *Am. J. Physiol. 254:* G81-G86.

LINDE, A. and GOLDBERG, M. (1993). Dentinogenesis. *Crit. Rev. Oral Biol. Med. 4:* 679-728.

LÖWKVIST, B., EMANUELSSON, H. and HEBY, O. (1983). Effects of polyamine limitation on nucleolar development and morphology in early chick embryos. *Cell Differ. 12:* 19-26.

LÖWKVIST, B., HEBY, O. and EMANUELSSON, H. (1980). Essential role of the polyamines in early chick embryo development. *J. Embryol. Exp. Morphol. 60:* 83-92.

LÖWKVIST, B., OREDSSON, S., HOLM, I., EMANUELSSON, H. and HEBY, O. (1987). Inhibition of polyamine synthesis reduces the growth rate and delays the expression of differentiated phenotypes in primary cultures of embryonic mesoderm from chick. *Cell Tissue Res. 249:* 151-160.

LUMSDEN, A.G.S. (1985). Contribution of the cranial neural crest in mammals. In *Tooth Morphogenesis and Differentiation* (Eds. A. Belcourt and J.V. Ruch). INSERM Vol. 125, Paris, pp. 29-40.

MAMONT, P.S., DUCHESNE, M-C., GROVE, J. and BEY, P. (1978). Anti-proliferative properties of DL-α-difluoromethylornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. *Biochem. Biophys. Res. Commun. 81:* 58-66.

MARK, M.P., BLOCH-ZUPAN, A. and RUCH, J.V. (1992). Effects of retinoids on tooth morphogenesis and cytodifferentiations *in vitro*. *Int. J. Dev. Biol. 36:* 517-526.

METCALF, B.W., BEY, P., DANZIN, C., JUNG, M.G., CASARA, P. and VEVERT, J.P. (1978). Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C.4.1.1.17) by substrate and product analogues. *J. Am. Chem. Soc. 100:* 2551-2553.

MORGAN, D.L. (1989). Polyamine oxidases and oxidised polyamines. In *The Physiology of Polyamines*. (Eds. U. Bachrach and Y.M. Heimer) Vol. I. CRC Press, Boca Raton, pp. 203-230.

OREDSSON, S.M., ANEHUS, S. and HEBY, O. (1980). Irreversible inhibition of the early increase in ornithine decarboxylase activity following growth stimulation is required to block Ehrlich ascites tumor cell proliferation. *Biochem. Biophys. Res. Commun. 94:* 151-158.

OREDSSON, S.M., KANJE, M., MAMONT, P.S., WAGNER, J. and HEBY, O. (1986). Polyamine depletion increases cellular ribonucleotide levels. *Mol. Cell. Biochem. 70:* 89-96.

PARTANEN, A. and THESLEFF, I. (1989). Growth factors and tooth development. *Int. J. Dev. Biol. 33:* 165-172.

PATHAK, S.N., PORTER, C.W. and DAVE, C. (1977). Morphological evidence for an antimitochondria action by methyglyoxal bis-(guanylhydrazone). *Cancer Res. 37:* 2246-2249.

PEGG, A.E. (1986). Recent advances in biochemistry of polyamines in eukaryotes. *Biochem. J. 234:* 249-262.

PEGG, A.E. (1988). Polyamine metabolism and its importance in neoplastic growth and as target for chemotherapy. *Cancer Res. 48:* 759-774.

PORTER, C.W., MILLER, J. and BERGERON, R.J. (1984). Aliphatic chain length specificity of the polyamine transport system in ascites L1210 leukemia cells. *Cancer Res. 44:* 126-128.

RATH, N.C. and REDDI, A.H. (1981). Changes in polyamines, RNA synthesis, and cell proliferation during matrix-induced cartilage, bone, and bone marrow development. *Dev. Biol. 82:* 211-216.

RUCH, J.V. (1984). Tooth morphogenesis and differentiation. In *Dentin and Dentinogenesis*, Vol. 1 (Ed. A. Linde). CRC Press, Boca Raton, pp. 47-80.

RUCH, J.V. (1990). Patterned distribution of differentiating dental cells: facts and hypotheses. *J. Biol. Buccale 18:* 91-98.

RUCH, J.V., KARCHER-DJURICIC, V. and THIEBOLD, J. (1976). Cell division and cytodifferentiation of odontoblasts. *Differentiation 5:* 165-169.

RUCH, J.V., LESOT, H., KARCHER-DJURICIC, V., MEYER, J.M. and MARK, M.P. (1983). Epithelial-mesenchymal interactions in tooth germs: mechanisms of differentiation. *J. Biol. Buccale 11:* 173.193.

RUPNIAK, H.T. and PAUL, D. (1978). Regulation of the cell cycle by polyamines in normal and transformed fibroblasts. In *Advances in Polyamine Research*, Vol. 1 (Eds. R.A. Campbell, D.R. Morris, D. Bartos, G.D. Daves and F. Bartos). Raven Press, New York, pp. 117-126.

SEILER, N. and DEZEURE, F. (1990). Polyamine transport in mammalian cells. *Int. J. Biochem. 22:* 211-218.

SEILER, N. and HEBY, O. (1988). Regulation of polyamines in mammals. *Acta Biochim. Biophys. Acad. Sci. Hung. 23:* 1-36.

SEILER, N., KNÖDEN, B., BINK, G., SARHAN, S. and BOLKENIUS, F. (1983). Diamine oxidase and polyamine catabolism. *Adv. Polyamine Res. 4:* 135-154.

SLOTKIN, T.A., SEIDLER, F.J., WHITMORE, W.L., WEIGEL, S.J., SLEPETIS, R.J., LEREA, L., TREPANIER, P.A. and BARTOLOME, J. (1983). Critical periods for the role of ornithine decarboxylase and the polyamines in growth and development of the rat: effects of exposure to α-difluoromethylornithine during discrete prenatal or post-natal intervals. *Int. J. Dev. Neurosci. 1:* 113-127.

STJERNBORG, L. and PERSSON, L. (1993). Stabilization of S-adenosylmethionine decarboxylase by aminoguanidine. *Biochem. Pharmacol. 45:* 1174-1176.

SUNKARA, P.S., PAGAC, M.B., NISHIOKA, K. and RAO, P.N. (1979). Differential effects of inhibition of polyamine biosynthesis on cell cycle traverse and structure

of the prematurely condensed chromosomes of normal and transformed cells. *J. Cell Physiol. 98:* 451-458.

TABOR, H. and TABOR, C.W. (1984). Polyamines. *Annu. Rev. Biochem. 53:*749-790.

TAKANO, T., TAKIGAWA, M. and SUZUKI, F. (1981). Role of polyamines in expression of the differentiated phenotype of chondrocytes in culture. *Med. Biol. 59:* 423-427.

THESLEFF, I., VAAHTOKARI, A. and VAINIO, S. (1990). Molecular changes during determination and differentiation of the dental cell lineage. *J. Biol. Buccale 18:*179-188.

VILLANUEVA, V.R. (1983). Are polyamines useful markers for monitoring cancer therapy? *Nato Adv. Sci. Inst. (Ser. A) 57:* 243-249.

WILLIAMS-ASHMAN, H.G. and PEGG, A.E. (1981). Aminopropylgroup transferts in polyamine biosynthesis. In *Polyamines in Biology and Medicine* (Eds. D.R. Morris and L.J. Marton). Dekker, New York, pp. 43-72.

WILLIAMS-ASHMAN, H.G. and SCHENONE, A. (1972). Methylglyoxal bis-(guanylhydrazone) as a potent inhibitor of mammalian and yeast S-adenosylmethionine decarboxylase. *Biochem. Biophys. Res. Commun. 46:* 288-295.

WOLPERT, L. (1978). Pattern formation in biological development. *Sci. Am. 239:* 124-135.

Accepted for publication: March 1995