Growth hormone and insulin-like growth factor-I in odontogenesis

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ABSTRACT This review documents recent insights into the roles of growth hormone and insulin-like growth factor-I during tooth formation. Hereditarily growth hormone-deficient Lewis dwarf rats and hypophysectomized rats have been used to document the influence of growth hormone on growth of the rat incisor and molar teeth *in vivo*. Cell population studies using bromodeoxyuridine labeling have shown that growth hormone administration to dwarf rats affects odontogenic cell proliferation in the incisor teeth. Immunohistochemistry, employing well-characterized monoclonal antibodies directed against the hormone, its binding protein/receptor, the growth factor and its receptor, has enabled the location of these proteins to be mapped in the ontogenic sequences of ameloblasts, odontoblasts and cementoblasts. This mapping is consistent with the concept that differentiating odontogenic cells are targets for the hormone and that insulin-like growth factor I is implicated as a secondary messenger in the same differentiating cell populations. The content of predentine and precementum matrices proteoglycans appears to be growth hormone-dependent. The proteoglycans implicated so far are rich in chondroitin sulphate and thus they may also be insulin-like growth factor I (sulphation factor)-dependent. Thus matrix synthesis may be what is principally affected by growth hormone in odontogenesis although no evidence of an effect on enamel matrix synthesis or proteoglycan content has yet been documented.

KEY WORDS: *growth hormone, amelogenesis, dentinogenesis, cementogenesis*

Growth hormone and insulin-like growth factor-1 in odontogenesis: background

Human studies

Children suffering pituitary dwarfism due to deficient growth hormone (GH) secretion have hypodontia, microdontia, permanent teeth fail to erupt and bone growth is retarded (Drews, 1971; Hamori *et al.*, 1974; Kosowicz and Rzymski, 1977). The bone changes are partially reversible by anabolic steroid therapy, although no significant improvement of dental growth has been reported (Kosowicz and Rzymski, 1977). Administration of GH to humans has been reported to stimulate growth and development of the jaws and hasten dental maturation in dwarfs with hypopituitarism (Kosowicz and Rzymski, 1977). The success of this treatment depends on the age at which therapy begins and the duration of the therapy (Sarnat *et al.*, 1988).

Bigeard and Sommermater (1991) studied 27 young people aged 3.5-17.5 years suffering from pituitary dwarfism. The crowns of affected premolar teeth were significantly smaller than normal, while the other teeth were of normal size. Partial eruption of the permanent dentition or late selective eruption of the premolar teeth, whose roots appeared normal, was also found. The hormone deficiency was also found to produce a decrease in the level of circulating growth factors, and particularly of epidermal growth factor (EGF). Human tooth histogenesis and morphology are probably dependent on the actions of GH on tooth cell proliferation and differentiation. Similar tooth anomalies are found in Laron-type dwarfism (Sarnat *et al.*, 1988). This type of dwarfism has been linked to deficiencies of the GH receptor/binding protein (GHrbp). Although the patients have a normal or high serum level of GH, they lack hepatic GH receptors and therefore suffer an IGF-I deficiency, secondarily. Dental development is less affected than skeletal maturation, and corresponds to chronological age being mostly manifest as reduced tooth crown size.

With respect to cementogenesis being affected by GH in humans, Snyder (1984) has reported an increase in the deposition of cementum in the teeth of individuals suffering pituitary gigantism.

Animal studies

The early studies which linked GH with odontogenesis were, in the main, performed on hypophysectomized [HYPOX] animals. Consequently the effect of GH deficiency could not be readily

Abbreviations used in this paper: BrdU, bromodeoxyuridine; EGF, epidermal growth factor; FCS, fetal calf serum; GalNAc N-acetyl galactosamine; GH, growth hormone; GHr, growth hormone receptor; GHrbp, growth hormone receptor/binding protein; HERS, Hertwig's epithelial root sheath; HPA, *Helix pormatia* agglutinin; HYPOX hypophysectomized; IGF-I, insulin-like growth factor-I; IHC, immunohistochemistry.

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separated from deficiencies of other trophic hormones. Moreover, the durations of these experiments studied the effects of long-term pituitary hormone deficiencies which were accompanied by profound effects on the overall metabolism of the animals. Schour and Van Dyke (1931a) found a measurable decrease in the growth rate of the continuously erupting rat incisor after hypophysectomy. The effects on the dental tissues were marked many months after operation. Gross findings were those of retardation and cessation of eruption. The incisor teeth, which were two thirds their normal size, were distorted in form, especially at their forming apical tip. The dentine being formed at the apical tip was buckled and the dental organ was irregular (Schour and Van Dyke, 1931b,c, 1934). In the long term, hypophysectomy diminished enamel formation, with ameloblast differentiation apparently being affected. At the histological level, enamel structure is reported to be irregular and poorly calcified, in long-term HYPOX rats (Schour and Van Dyke, 1931a).

Prolonged administration of GH to intact animals also apparently affects the life cycle of ameloblasts. Becks *et al.* (1948) reported that whereas in normal rats, incisor enamel formation occurs in a restricted zone, ameloblasts appear "active" almost to the junction with the oral epithelium in rats which had received GH for almost a year. It is difficult to interpret the meaning of this observation in terms of what is now known about the life cycle of ameloblasts. No discrimination was made by these authors between the secretory and maturation stages of ameloblasts or of the reduced enamel epithelium. Whether any control was employed on the orientation of the longitudinal sections of the incisors to ensure that comparable zones of ameloblasts were compared was not stated. Nonetheless, it is an interesting observation which might mean that the size of the compartments in ameloblast life cycle might be altered by the prolonged administration of GH in excess of physiologic blood levels.

Growth hormone has been shown to restore dentine production in the continuously erupting incisor of HYPOX rats, whereas other pituitary hormones are without effect (Hansson *et al.*,1978a,b). Several lines of evidence suggest that cementum is a target tissue for the hormone. In the molar teeth of HYPOX rats cementum formation is diminished although some hyperplasia of cementum is observed at the cemento-enamel junction (Schour and Van Dyke, 1931a, 1934). Replacement therapy with GH increases cementum formation at the apices of molar roots (Collins *et al.*, 1949). Becks *et al.* (1948) observed considerable hypercementosis on the roots of molar teeth of intact rats given GH daily for 14 months. Similar responses of cementoblasts to GH in HYPOX rat molar teeth have been documented by Baume *et al.* (1954) and Ratcliffe and Oliver (1966).

Recent studies

Knowledge of the receptor which mediates the somatic and metabolic activities of GH has recently been greatly expanded by two key developments: cloning of the GH receptor (Leung *et al.*, 1987) and characterization of monoclonal antibodies to the receptor (Barnard *et al.*, 1984, 1985). I have been fortunate to be associated with Dr. Michael Waters, Department of Physiology and Pharmacology, the University of Queensland, who among other things has reported a 30% sequence homology between the GH receptor and the prolactin receptor (Waters *et al.*, 1988, 1990). With the concurrent cloning of a number of hematopoetic receptors, the GH receptor has been found to be structurally related to a major class of cytokine receptors which are involved in cellular proliferation, commitment and differentiation (Bazan, 1989; Cosman *et al.*, 1990). In the broadly based studies of Waters on the structure, location and role of the GH receptor, I have been privileged to have contributed insights derived from the dental tissues (for review see Waters *et al.*, 1992). The dental tissues, enamel, dentine and cementum are unique tissues for investigating cellular proliferation, commitment and differentiation of the cells of calcified tissues both in the fetus and in the adult. Specifically, the continuously erupting rat incisor is a unique model in which an integration of cell proliferation, cell commitment and differentiation contribute to persistent growth which may be regulated by GH through its receptor and which conceivably involves insulin-like growth factor-I (IGF-I) and its receptor in secondary mediation of these effects.

We have used the rat incisor model in the selectively GH-deficient Oxford dwarf rat (Charlton *et al.*, 1988). This animal has the advantage over the HYPOX rat in having only 5% of the normal output of pituitary GH, with all other pituitary hormones being expressed at normal levels (Charlton *et al.*, 1988). We have also conducted limited studies on the incisors and molars of HYPOX rats and, in all of these tissues, we have employed the well-characterized monoclonal antibodies provided by Waters and his associates with standard immunocytochemical techniques to characterize the location of GH, growth hormone receptor/bindingprotein (GHrbp), the intracellular moeity of the GH receptor (GHr), insulin-like growth factor-I (IGF-Ir) and its receptor, both in fetal and adult rat tissues. The induction of dental tissue matrix proteoglycans by GH has been investigated by specific lectin-binding and immunohistochemistry (IHC).

A further recent development has been a fruitful collaboration with Professor Jean Victor Ruch of the Institut Biologie Médicale, Strasbourg, in which we have commenced studies of the effects of GH and IGF-I on mouse molar tooth buds *in vitro*.

This work is as yet unpublished. References to the publications which have emanated from these collaborations are made in this review. I have been privileged to have worked with many talented people in this endeavor and my debt is hereby acknowledged. I hope that by bringing together my thoughts on the effects of GH and IGF-I on odontogenesis as I presently see them, that this synthesis will be both an acknowledgement of our mutual endeavors and a stimulus to further dialogue.

Growth hormone and insulin-like growth factor-1 in amelogenesis: population dynamics

To determine what effect GH might have on the population dynamics of the odontogenic epithelia, the bromodeoxyuridine (BrdU) labeling indices, mitotic indices, and cell compartment sizes of the several odontogenic epithelia of the rat incisor (Fig. 1) were determined (Young *et al.*, 1992). Dwarf animals were treated with twice daily injections of GH for six days and compared with normal and dwarf rats which received equivalent injections of normal saline. All were killed 7 h after the final injection of GH, and 2 h after an injection of BrdU. Thus, the measured effects on the ameloblast population in dwarf rats were subsequent to several days of GH exposure; and the cells in DNA synthesis or mitotic phase could reasonably have been affected by influences from the final GH injection of growth hormone.

Differences were indeed found in the labeling and mitotic indices of the inner enamel epithelium, the stratum intermedium, and in the epithelium of Hertwig's epithelial root sheath (HERS),

between normal and dwarf rats. The latter group had significantly lower than normal indices. Moreover, these indices were equivalent to normal in dwarf rats treated with growth hormone. Pipeline effects of higher indices were appreciable in expansions of cell numbers in compartments, notably in the preameloblast compartment overlying dentine matrix on the labial aspect of the tooth. Thus we were able to report, for the first time, that GH appears to affect the population dynamics of the odontogenic epithelia.

What is the mechanism whereby GH affects odontogenic epithelial cell proliferation? *In vitro* studies of the effect of GH on the mitotic rate of the inner enamel epithelium of mouse tooth buds in serum free medium show that there is evidence that odontogenic epithelial stem cells are targets for GH, as a mitogen (unpublished) (Table 1). However, as described in the next section, growth hormone receptor/binding (GHrbp) protein, is not demonstrable by IHC in these embryonal cell types of the epithelial diaphragm. Although, curiously, GHrbp was demonstrable in such cells during mitotic phase *(vide infra)*. Insulin-like growth factor-1, and its receptor, are likewise not demonstrable by immunohistochemistry in these embryonal cell types (Joseph *et al.*, 1993, 1994a) making it unlikely that the GH mitogenic stimulus is mediated through autocrine/paracrine IGF-1 in this population. An intriguing alternative mitogen may be epidermal growth factor (EGF), for we have demonstrated changes in the expression of EGF receptor within the epithelial diaphragm and the mesenchymal cells at the interface between the dental papilla and the dental follicle, when the distribution of staining for EGFr is compared between normal, dwarf and GH-treated dwarf rats (unpublished). If the expression of EGF receptor (EGFr) by such odontogenic stem cells could be shown to be responsive to altered GH status, EGF, which is known to maintain cells in an undifferentiated state, might also act as a GH-dependent mitogen. Further if this GH-EGFr-EGF connection could be established, some of the effects of GH on tooth eruption might be elucidated.

An alternative mechanism whereby GH might influence DNA synthesis and mitotic activity in these stem cell populations may lie in some feedback mechanism resulting from differentiation. Thus, if GH promotes the odontogenic cells to differentiate, feedback would induce the stem cells to divide to maintain the pipeline. Presumably, a similar feedback mechanism could operate when mitotic activity is stimulated in the odontogenic stem cells of the rat incisor by mechanical reduction of the incisor crown length (Zajicek *et al.*, 1972). Insulin-like growth factor-1 does appear to influence the mitotic rate of inner enamel epithelium in mouse molar tooth buds *in vitro* in serum-free medium, but not as effectively as fetal calf serum or GH (unpublished) (Table 1).

Growth hormone and GH receptor distribution

GH, GHr, IGF-I and IGF-I receptor distribution as they are demonstrable by IHC in the odontogenic epithelium when it changes through its histogenetic to its histodifferentiation stage will now be discussed. The expressions of these molecules appears to be

TABLE 1

COMPARISON OF VARIOUS PARAMETERS IN MOUSE MOLAR TOOTH BUDS

	Control	+FCS	GH50	GH100	IGF100	IGF200
Mean volumes						
day 4	0.02686	0.08631	0.05319	0.0483	0.0457	0.09699
day 5	0.02686	0.09156	0.04139	0.0513	0.06027	0.11865
day 6	0.02727	0.07154	0.04668	0.04806	0.1055	0.14858
Significance	N.S.	N.S.	N.S.	N.S.	SIG	SIG.
-within treatments					p<0.05	p<0.05
					-0.01	
-between treatments	IGF100+FCS		IGF100+FCS	IGF100+FCS	+FCS	
	IGF200	IGF200	IGF200	IGF200	IGF200	
Differentiation						
day 4	0	0	0	0	0	2
day 5	0	0	0	0	5	6
day 6	0	2	0	2	6	6
Mitotic indices						
(per 100 cells)	0.2	1.88	0.61	0.42	0.68	0.97
Papilla cell density	0.175	0.174	0.241	0.224	0.165	0.145
Significance	GH50	GH50			GH50	GH50
between treatments		GH100			GH100	GH100

Comparison of various parameters in mouse molar tooth buds grown in serum-free (CONTROL), fetal calf serum-rich (+FCS) or in serum-free conditions with the following supplements: growth hormone 50 ng/m. (GH50) or 100 ng/ml (GH100), insulin-like growth factor-1 at 100 ng/ml (IGF100) or insulin-like growth factor-1 at 200 ng/ml (IGF200). The following parameters were measured: *mean volumes,* volumes are in cubic millimeters of mouse molar tooth buds after 16 post-conception plus 4,5 and 6 days *in vitro* treatment. Buds of greatest volume were found after 6 days in medium containing IGF-1 at 200 ng/ml; *significant volume increases,* within treatments, both insulin-like growth factor-1 treatments showed significant growth over the three days of treatment (ANOVA). Between treatments differences were significant at p<0.05-0.01; *differentiation,* maximum differentiation was achieved, by the number of tooth buds given, when a definitive band of dentin was produced and preameloblasts became tall, columnar cells with proximally polarized nuclei. Over the three days of culture IGF-1 groups produced the maximum differentiation in the greatest number of tooth buds; *mitotic indices,* the mean number of mitoses counted in the early preameloblasts/preodontoblasts in the epithelial diaphragm area of the buds (per 100 cells). Growth hormone treatment appears to have been approximated the mitotic activity of FCS; *papilla cell density,* the mean number of cells per square micrometer in the papilla at day 16+6. Also shown are the treatments that have a significantly higher density as determined by ANOVA.

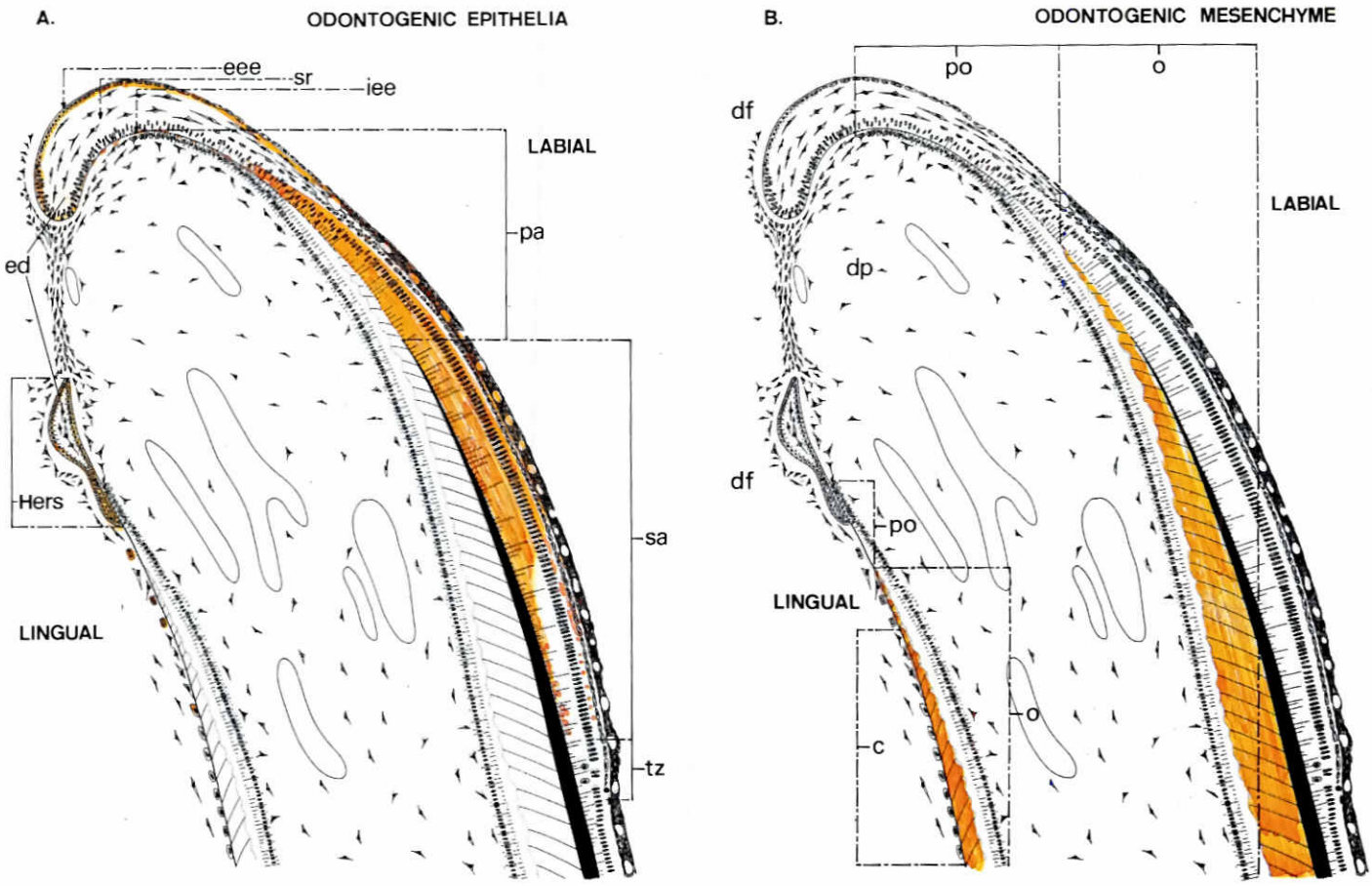

Fig. 1A. Diagram showing the locations and layering of the odontogenic epithelial populations in the apical third of the rat incisor. *At the apical foramen are depicted two sections of the epithelial diaphragm (ed) where, on the labial aspect morphogenesis commences with differentiation of the external enamel epithelium (eee), the stellate reticulum (sr) and the inner enamel epithelium (iee). On the lingual aspect, morphogenesis is delineated by Hertwigs epithelial root sheath (Hers). Differentiation of odontoblasts and dentinogenesis occur adjacent to the iee and to Hers. The inner enamel epithelium differentiates to the phenotype of preameloblasts (PA) which initially overlie nonpolarized cells of the dental papilla. While polarization of preodontoblasts occurs and dentine matrix (hatched) is laid down, the preameloblasts retain the capacity to divide, until the onset of enamel matrix secretion by the secretory ameloblast phenotype (sa). When enamel matrix (solid black line) has been formed to its thickest extent, the ameloblasts undergo a transition in a short zone (tz) to the maturation phenotype. This transition zone is marked by apoptotic bodies.*

Fig. 1B. Diagram illustrating the locations and layering of the odontogenic mesenchymal populations in the apical third of the rat incisor. *At the apical foramen is depicted the interface of densely-packed mesenchymal cells between the dental follicle (df) on the outer aspect of the epithelial diaphragm and the dental papilla (dp) on its inner aspect. On the labial aspect, mesenchymal cells of the dental papilla polarize against the inner enamel epithelium as preodontoblasts (po), and subsequent differentiation to odontoblasts (o) is evidenced by dentine (hatched). Between the odontoblast layer and the dentine, the predentine layer is indicated by a clear zone of uniform width. On the lingual aspect odontoblasts differentiate in association with Hertwigs epithelial root sheath which subsequently breaks down permitting dental follicular mesenchymal cells to approach the lingual dentine to differentiate into cementoblasts (c) which attach the collagen fibres of the periodontal ligament to the tooth by the formation of acellular cementum.*

closely linked to differentiated function of the cells rather than to their mitotic activity.

One of the possible objections to the concept that GH is essential to odontogenesis might be that fetal pituitary GH is unlikely to be available to the developing tooth germs *in utero* due to the embryonal state of the pituitary up until when tooth bud formation is already underway. However an IHC investigation of GH distribution in rat molar tooth buds enabled us to demonstrate GH distributed in the several locations in odontogenic epithelia (unpublished). This opens up the intriguing possibility that a GH-like molecule is produced in fetal tissues outside the pituitary and specifically in the odontogenic tissues. Thus, within the odontogenic epithelia of prenatal rat molars the localization of GH, GHrBP and the cell membrane-associated GHr has been investigated and has been found associated with fetal odontogenesis.

The distribution of GHrBP has been mapped within the dental organ of the normal rat incisor by IMC (Zhang *et al.*, 1992a). As indicated above, undifferentiated cells of the epithelial diaphragm, including HERS do not show staining for GHrBP. However, polarized preameloblasts of the inner enamel epithelium are the first cells of the ameloblast lineage to display immunoreactivity. This is found in the distal cytoplasm of these cells adjacent to the basement membrane of the future amelodentinal junction. At this stage of histogenesis, the mesenchymal cells of the subadjacent dental papilla are not yet polarized. This distribution supports the concept that as

preameloblasts embark on cyto-differentiation they become targets for the action of the hormone.

This concept was further supported by the finding that the nucleolar organizer region (NOR) protein expression, at this stage of preameloblast differentiation, was responsive to GH when evaluated with the specific silver staining (AgNOR technique) and measurement by microphotometry (Young and Zhang, 1989; Zhang *et al.*, 1992b) comparing normal, HYPOX and HYPOX rats which had received GH. This technique is a measure of ribosomal RNA (rRNA)synthesis. Which rRNAs are being synthesized during the histogenesis phase by the preameloblast is unknown. However, as long as the preameloblast population remained mitotically active, the AgNOR regions were susceptible to the influence of GH. Post-mitotic ameloblast AgNOR did not appear to alter with changes in the GH status of HYPOX animals.

Insulin-like growth factor-I and its receptor distribution

Insulin-like growth factor-I has been demonstrated by IHC in the early stages of rat molar tooth bud development *in vivo* (Joseph *et al.*, 1994c). This localization of IGF-I immunoreactivity correlates with the distribution of GHrbp in the same tissues at different stages of tooth development (Joseph *et al.*, 1994b). Thus GH may be the major regulator of IGF-I expression in the early stages of tooth development particularly as GH, GHrbp and the intracellular GH-receptor epitope can be demonstrated in fetal molar tooth buds *(vide supra).*

In the rat incisor, the preameloblasts, as they undergo histodifferentiation overlying dentine matrix, are the first cells of the inner enamel epithelial cell life cycle to demonstrate immunohistochemical staining for IGF-I (Joseph *et al.*, 1993). The intensity of this staining for IGF-I has been measurably linked to GH administration in dwarf rats (Joseph *et al.*, 1993). While this has not so far been demonstrated by *in situ* hybridization, it is probable that ameloblast IGF-I mRNA expression is GH-dependent in the ameloplast cell lineage.

In this connection, it is interesting that IHC distribution of IGF-I receptor staining in ameloblasts has been shown to change over the life cycle of ameloblasts (Joseph *et al.*, 1994b). During early enamel secretion, diffuse staining for IGF-I receptor is found in secretory stage ameloblasts. However, during the late secretory stage, the diffuse staining changes to an increasingly granular pattern of IGF-Ir staining in the cytoplasm of ameloblasts in this stage of their life cycle. This granular positivity gradually becomes confined to the distal cytoplasm of the ameloblasts in the late secretory stage. When the full thickness of enamel matrix has been formed, the ameloblasts undergo a dramatic change in their morphological phenotype from that of a highly specialized enamel matrix protein-secreting cell to that of cell engaged in matrix maturation. Maturation ameloblasts exhibit alternating patterns of modulation between ruffled and smooth-ended forms when viewed ultrastructurally. This change in phenotype takes place over a transitional zone in the life cycle of the ameloblasts (Fig. 7). This transitional stage has been estimated to occur over a 15-20 h period within the 30 day transit time of the ameloblast life cycle, with the cells in the transitional zone occupying 0.5 mm of the labial surface of the forming enamel (Leblond and Warshawsky, 1979). Remarkably, the cells undergoing this transition in phenotype do not stain for IGF-I-r. However, with the adoption of the modulating phenotypes of maturation, ameloblasts again exhibit strong diffuse staining for the IGF-Ir. Much could be learned, for example about the varying relationships of the receptor to cell organelles at the ultrastructural level, when at the light level findings are of a change from a diffuse to a granular staining pattern. Is this an example of endocytotic membrane-receptor processing? What is the sequence of IGF-I receptor synthesis when the cells embark on enamel maturation after emerging from the transitional state? This curious sequence of events, with respect to the distribution of IGF-Ir in the ameloblast life cycle, is clearly worthy of further investigation.

It should also be noted, in passing, that ameloblasts make the transition, from the secretory to the maturation phenotype, without intervening mitotic activity. The signal for this transition is unknown, but it appears to be preceded by a gradual downregulation of IGF-I receptor expression. Presumably this, in turn, renders the cells in the transitional stage refractory to IGF-I stimulation. Accordingly, it is fascinating to learn that the transitional zone is characterized by programmed cell death which affects a proportion of the ameloblasts, estimated at 50% (Smith and Warshawsky, 1975). Morphologically, apoptosis is programmed cell death characterized by cells undergoing both nuclear and cytoplasmic condensation that results in the formation of apoptotic bodies which are then phagocytosed by neighboring cells (Kerr and Harman, 1991). Pyknotic nuclear fragments are found in the transitional zone which, ultrastructurally, show all of the criteria for apoptotic bodies (Smid *et al.*, 1990; Kerr and Harman, 1991). Joseph *et al.* (1994a) have shown, by *in situ* hybridization, that the mRNA for sulphated glycoprotein-2 (SGP-2), a marker protein for apoptosis, is specifically expressed over the transitional zone. Accordingly, it is tempting to speculate that apoptosis of a proportion of the transitional ameloblasts is one further example in developmental biology of programmed cell death associated with a depletion of endocrine stimulation. In this case it would be a depletion of IGF-I, predicated upon by downregulation of its receptor.

Enamel matrix changes

The relative influences of GH and IGF-I on enamel organ differentiation can best be appreciated in organ culture of mouse molar tooth buds (unpublished). The relative effects of GH and IGF-I and fetal calf serum (FCS) were compared with serum-free medium as to their effects on odontogenesis in 16-day fetal mouse first molar tooth buds. The study compared volumetric changes in tooth buds between three days of treatment (4, 5, 6 days *in vitro*). All three treatments, GH, IGF-I and FCS, produced greater volume in tooth buds than the serum-free medium. Insulin-like growth factor I produced greater volumes than all other treatments (Table 1). In this tissue culture model, dentine matrix, but not enamel matrix, is formed. The preameloblasts become postmitotic and differentiate, overlying the dentine matrix to become highly polarized tall columnar cells, supported by a well-developed statum intermedium and stellate reticulum. When the attainment of this degree of differentiation was compared between days and between treatments, GH treatment appeared to induce the postmitotic differentiation of preameloblasts with the same frequency, and at the same rate over the 3 days, as did FCS. In contrast, IGF-I induced these change *in vitro* as early as 4 days and in all buds by 6 days (Table 1). These results suggest that factors in serum including GH and IGF-I are important for enamel organ differentiation, and that IGF-I may be particularly important in this respect.

However, we have so far been unable to document any appreciable influence of GH or IGF-1 on any extracellular components of enamel matrix. A specific lectin-binding study in the dwarf rat

incisor model failed to demonstrate differences in staining distribution or intensity in the enamel organ of any of the panel of lectins employed (Zhang *et al.*, 1994). Unlike predentine, which shows differences between normal, dwarf and growth hormone-treated dwarf animals with *Helix pomatia* lectin specific for N-acetylgalactosamine (GalNAc), immature enamel matrix does not stain with this lectin. The cytoplasm of secretory ameloblasts was positive for GalNAc, but this positivity did not appear to alter between the treatments.

An IHC study, again in the dwarf rat incisor model, using antibodies directed against two GalNAc-containing proteoglycans, biglycan and decorin, likewise failed to demonstrate staining of the enamel matrix (unpublished). Staining for biglycan was absent in all of the odontogenic epithelia. Decorin was localized in the inner and external enamel epithelia but not in the stellate reticulum. However, the staining for decorin in the external enamel epithelium and in its derivative, the papillary layer, was particularly well-delineated in the normal but not in the dwarf rats. Growth hormone-treated dwarf animals showed positivity equivalent to normal in the external enamel epithelium. Decorin is known to act as a sink for transforming growth factor ß. This finding suggests that the interface between the external enamel epithelium/papillary layer and the dental follicle is a site where growth factor interactions may be involved in maintaining the unique qualities of the vascular supply to ameloblasts and that the expression of decorin at that site is GH-dependent.

In summary, these studies of the population kinetics, of hormone, growth factor and receptor distribution and of expressions of differentiation have yielded tantalizing indicators that GH and IGF-I play roles in the regulation of growth in the odontogenic epithelia.

Growth hormone and insulin-like growth factor-1 in dentinogenesis: population dynamics

The interfaces between the odontogenic epithelium, the mesenchyme of the dental papilla and of the dental follicle are sites of intense cell proliferation and histogenesis. The mesenchymal cells which synthesize DNA and divide are destined to differentiate into odontoblasts and pulp cells, from within the dental papilla centrally (Chiba, 1965; Chiba *et al.*, 1967; Robins, 1967), and peripherally within the dental follicle, into cementoblasts and periodontal ligament fibroblasts (Cho and Garant, 1988). The mesenchymal cells of the dental papilla become separated from the peripheral cells of the dental follicle by the growth intrusion of the odontogenic epithelium of the epithelial diaphragm. Both the labial inner enamel epithelium and the lingual HERS interface with the central dental papilla where two populations of preodontoblasts can be observed and measured as to their DNA synthesis and mitotic activity prior to postmitotic differentiation into odontoblasts. Ruch (1990) has suggested that these populations enter a finite number of cell cycles prior to differentiation, which thus controls the number of postmitotic odontoblasts. This in turn would determine the amount (length) of dentine and hence the shape of the tooth. However differences in the indices of cell proliferation between the labial and lingual preodontoblast populations, which might have been expected from the greater curvature of the labial dentine, were not found.

Accordingly, we hypothesized that if GH affects the cell dynamics of the preodontoblast population, it might be shown to produce increased BrdU labeling indices, increased mitotic indices and increased populations of odontoblasts active in matrix secretion prior to the onset of amelogenesis. Labeling (BrdU) and mitotic indices were determined on a per area basis within the dental papillae and on a per/100 cell basis in the labial and lingual preodontoblast populations. Differences were appreciated between the labial and lingual populations as expected above (Young *et al.*, 1993). The labeling and mitotic indices of all of the odontogenic mesenchymal cell populations are significantly lower in dwarf rats than in normal rats. Administration of GH to dwarf rats produces labeling and mitotic indices equivalent to those of normal rats. The effect of GH administration for six days is also appreciable in the significant increase in total cells in the preodontoblast compartments both labially and lingually. Moreover, comparison of the lengths of predentine-dentine matrix formed, before the onset of enamel matrix secretion, revealed that less dentine matrix was formed by dwarf rats in 6 days than by normal rats. Thus, 6 days of GH administration produces dentine matrix in dwarf rats equivalent to that in normal rats.

This suggests that the effect of GH on dentine growth in dwarf rats is to produce a larger population of odontoblasts, subsequently active in dentine matrix deposition. These are derived from an enlarged population of preodontoblasts dividing both within the preodontoblast layer and derived from the dental papilla. The undifferentiated cell population of the dental papilla is also responsive to the GH status of the animal. However, if dentine matrix differentiation and synthesis are coupled to preodontoblast cell division by as yet poorly understood feed-back mechanisms *(vide supra)*, it may be that these observed differences in odontogenic mesenchyme proliferation, with changes in GH status, are sequelae of the direct action of GH on odontoblast differentiation and dentine synthesis, rather than a direct mitogenic action of GH on the preodontoblast population.

Preliminary studies of odontogenic cell proliferation *in vitro* suggest that both GH and IGF-I support proliferation equivalent to fetal calf serum (Table 1). However, IGFI-treated cultured molar tooth buds exhibited greater degrees of differentiation (unpublished observations). Growth hormone enhances the number of mitotically active chondroblasts which are sensitive to IGF-I (Nilsson *et al.*, 1986). Green *et al.* (1985) have suggested that IGF-I acts as a progression factor for clonal expansion of only a small proportion of previously differentiated GH-primed chondroblasts. It may be that the influence of GH and IGF-I on the proliferation of the odontogenic mesenchyme is similar to that postulated for cartilage and bone, i.e. that both are mitogenic, but that GH primes a small proportion of preodontoblasts which then become IGF-I sensitive.

In general IGF-I is a relatively weak mitogen that enables competent cells to progress through the S phase of the cell cycle (Schmid and Ernst, 1991). The degree of mitogenic activity induced by IGF-1 may vary with cell type and environmental conditions (Rosen *et al.*, 1994). Both GH and IGF-I stimulate osteoblast-like cell proliferation *in vitro* in a dose-dependent manner. Insulin-like growth factor 1 is a strong mitogen for bone cells in fetal rat calvaria, as well as an influence on bone matrix formation (Hock *et al.*, 1988; McCarthy *et al.*, 1989). Moreover IGF-I rapidly increases the expression of mRNA for the oncogene *c-fos* which encodes for transactivation factors that are expressed during osteoblast proliferation (Merriman *et al.*, 1990; Stein and Lian, 1993). Thus other cytokines and growth factors may be involved in intercellular communication by which the apical proliferation of odontogenic cells is coordinated with growth and eruption of the rat incisor.

Growth hormone and its receptor distribution

The expression of GH, GHrbp and GHr in the dental papilla have been traced in fetal rats to the earliest stages of tooth bud development (Joseph *et al.*, 1994a and unpublished). Growth hormone can be detected by IHC as early as the cap stage within the dental papilla and its expression does not *appear* to vary with time. In general the undifferentiated dental mesenchymal cells of the dental papilla, up until the cap stage, showed very weak staining for GHrbp. However, with the onset of preodontoblast and odontoblast differentiation, these cell stages showed progressive expression of this antigen. It is a consistent finding that preodontoblast polarization and odontoblasts engaged in matrix formation are positive for GHrbp and GHr.

An IHC study of the distribution of GHrbp within the dental papilla of the rat incisor, and the odontoblast cell lineage in the rat molar, support the contention that GH is primarily concerned with enhancing odontoblast differentiation and matrix synthesis (Zhang *et al.*, 1992a,c). Growth hormone receptor/binding protein is not expressed in the odontoblast lineage in the rat incisor until there is evidence of histogenesis, i.e. demonstrable polarization of preodontoblasts. Moreover the intensity of staining increases with the formation of dentine matrix. Curiously, the staining of GHrbp is absent when odontoblasts have formed the full thickness of dentine at any one site (Zhang *et al.*, 1992c). We have found that within the pulp chambers of adult rat molars, odontoblasts on the coronal aspect of the pulp, which have formed the full thickness of dentine beneath that aspect, stain negatively for GHrbp; whereas odontoblasts forming the radicular aspect of the pulp chamber and the root apices are strongly positive. Similar staining contrasts are found between osteoblasts and osteocytes, cementoblasts and cementocytes, implying a change in GHrbp expression with diminished function in these equivalent mesenchymal cell ontogenic sequences.

A study of the specific silver-stained nucleolar-organizer regions (AgNOR) in the odontoblast cell lineage in HYPOX rats, with or without GH treatment showed that AgNOR, in preodontoblasts and odontoblasts, were less in extent in HYPOX rats and approach normal values with GH treatment (Zhang *et al.*, 1992b). Unlike post-mitotic ameloblasts, post-mitotic odontoblasts are responsive to GH as measured in this estimate of rRNA synthesis. This may explain why it has been easier to demonstrate changes in the amount of dentine formed under conditions of GH stimulation than to demonstrate changes in amount of enamel matrix (see Hansson *et al.*, 1978b).

Insulin-like growth factor-I and its receptor distribution

The distribution of IGF-I and its receptor in the odontoblast cell lineage has been documented by IHC in fetal rat molars and in the rat incisor (Joseph *et al.*, 1993, 1994a,b). Changes in the expression of IGF-1 by ameloblasts in response to GH treatment in the rat incisor have been shown in the dwarf rat model (Joseph *et al.*, 1993). The localization of IGF-I immunoreactivity correlates identically with the distribution of GHrbp expression in the different stages of fetal molar tooth development (Joseph *et al.*, 1994a) and, in the odontoblast ontologic sequence, identical expression of IGF-I and GHrbp are found. Interestingly, insulin-like growth factor-I receptor is found to be strongly expressed in predentine (Joseph *et al.*, 1994b) as well as by odontoblasts.

In vitro, it has been demonstrated that both GH and IGF-I replicate the effects of fetal calf serum on dentine differentiation in the mouse molar organ culture model (unpublished). In this system, in the absence of fetal calf serum, tooth buds fail to grow in volume, differentiation of dentinal matrix is poor and post-mitotic differentiation of ameloblasts does not occur. The addition of GH does not significantly increase the volumes of tooth buds grown in serum-free medium (Table 1). However, it does support the formation of dentine matrix and the post mitotic differentiation of ameloblasts equivalent to that found in the medium containing fetal calf serum. Insulin-like growth factor-I, when added to the medium, produces tooth buds of greater volume than either GH or FCS in which dentine differentiation occurred earlier and to a greater degree than with the other treatments (Table 1). These findings suggest that it is principally IGF-I which promotes the differentiation and development of odontoblasts and their differentiated cell function, i.e. predentine matrix formation. It is not clear to what extent these effects are mediated *in vivo* by GH, by systemic IGF-I or by locally up-regulated IGF-I, and the roles of their respective binding proteins and receptors on odontoblasts remain to be clarified. However, in view of the distribution and expression of GHrbp, IGF-I and IGF-Ir in preodontoblasts and functioning odontoblasts, it is likely that they are target cells for the hormone and the growth factor.

Dentine matrix changes

If GH acts through its receptor to up-regulate local expression of IGF-I in odontoblasts, as demonstrated by Joseph *et al.* (1993), it could, in turn, influence the glycosylation of dental matrix proteoglycans. Insulin-like growth factor-I was initially characterized by its capacity to effect sulphation of the matrix proteoglycans of cartilage (Daughaday *et al.*, 1972). Accordingly, we performed a study which employed a panel of lectins to map the distribution of specific sugars in the rat incisor model and to determine whether GH influenced the distribution of any of the sugars in the tissues of dwarf rats with or without hormone replacement (Zhang *et al.*, 1994).

The lectin-binding profiles of the odontoblasts and of predentine matrix of normal rats were found identical to those previously described by Nakai *et al.* (1985). However, in dwarf rats, the extent of staining in the predentine with *H. pomatia* lectin (HPA) specific for N-Acetyl galactosamine (GalNAc) was consistently reduced. This was restored to the extent found in normal rats when GH was administered for 6 days. As GalNAc is found specifically in the chondroitin sulphate-containing proteoglycans of predentine, it was postulated that GH might indeed regulate a sulphated proteoglycan or glycoprotein in dentinogenesis (Zhang *et al.*, 1994).

As the components in predentine which stain specifically by HPA may comprise a wide range of GalNAc containing glycoproteins or glycolipids, the same experiment was repeated using IHC with antibodies directed against the core proteins of two GalNAc-containing proteoglycans, i.e. biglycan and decorin (unpublished) The distribution of decorin paralled the distribution of GalNAc in all of the dental tissues. Although it was stained for weakly in the predentine of normal rats, it was minimally demonstrated in dwarf rat predentine, and was restored to normal extent in the GH-treated dwarf rats. Biglycan, on the other hand, stained only the predentine strongly in normal and GH-treated dwarfs and, to a much lesser extent, in untreated dwarf animals. As the biological functions of these proteoglycans is thought to relate to the regulation of assembly and growth of collagen fibers (Öbrink, 1973), such a role could be expected in predentine matrix. This would link GH to the growth of the matrix of dentine. Interestingly, neither GalNAc,

decorin, biglycan [or IGF-I receptor] were demonstrable by these methods in undecalcified or decalcified dentine.

Growth hormone and insulin-like growth factor-1 in cementogenesis: population dynamics

The use of BrdU labeling to study proliferation of precementoblasts in the lingual dental follicle of the rat incisor yields only limited information on the influence of GH on precementoblast proliferation among cells of the dental follicle (Young *et al.*, 1993). Mesenchymal cells of the area of the dental follicle adjacent to HERS labeled positively for BrdU but no differences were noted between normal, dwarf or GH-treated dwarf animals. In the zone distal from HERS, after it has disintegrated and where the mesenchyme of the follicle interfaces with dentine, more BrdU-positive mesenchymal cells per unit length were found in normal than in dwarf rats. Growth hormone did not, however, significantly affect the number of this population in dwarf rats. Presumably only a proportion of the follicular mesenchymal cells differentiate to become cementoblasts which form the acellular cementum against dentine, thus attaching the periodontal ligament (Cho and Garant, 1988). This differentiation occurs over a short length of this zone. Accordingly, it was not possible to state unequivocally that GH influenced the proliferation of precementoblasts which form acellular cementum.

The effect of GH on the proliferation of precementoblasts prior to the formation of cellular cementum, as found on the rat molar, has likewise not been elucidated. However in HYPOX animals, precementoblast proliferation does seem to be affected by GH administration, as a greater number of cells per unit length of cellular cementum were found in HYPOX animals treated with GH for 10 days, than were found in normal or HYPOX rats (Clayden *et al.*, 1994). These findings suggest that the precementoblasts of cellular cementum may be sensitive and respond to GH by proliferation.

Growth hormone and its receptor distribution

The expression of GHrbp by the cementoblast lineage at various stages shows a similar pattern to that found in odontoblasts and in osteoblasts, i.e. that GHrbp can be demonstrated by IHC on precementoblasts and cementoblasts actively forming cellular cementum, but not on cementocytes (Zhang *et al.*, 1992c). However, we have been unable to demonstrate GHrbp on those cementoblasts associated with acellular cementum, either on rat incisors or on molar teeth. Moreover, while GH can be shown to affect cellular cementum matrix formation, we have been unable to demonstrate measurable effects on acellular cementum formation (Clayden *et al.*, 1994). This may only be due to the paucity of this material or a lack of discrimination of the methods. However it has been suggested that acellular cementum is formed by a cell of the periodontal ligament which does not exhibit the full expression of the cementoblast phenotype (Bosshardt and Schroeder, 1990) and thus possibly is not GH responsive.

Insulin-like growth factor I and its receptor

Little information is as yet available on the distribution of IGF-I or its receptor in the cementoblast lineage.

Cementum matrix changes

There is evidence that GH plays a role in stimulating intracellular protein translation by cementoblasts and matrix formation. This derives from our ultrastructural study of the influence of hypophysectomy and GH treatment on rat molar cementogenesis (Clayden *et al.*, 1994). At 60 days after birth, the third maxillary molars of normal rats are in active eruption and, at the apices of both of their roots, dentine formation and active cementogenesis are completing the normal length and width of the root. The roots, on their coronal two thirds, are covered by acellular cementum, while cellular cementum is being formed at the apical thirds. This distinction is important, for it appears that it is cellular cementum which is measurably affected by hypophysectomy and responsive to GH. Three weeks after removal of the pituitary in 35-day-old rats, measurably less cellular cementum was formed on the third molar roots of HYPOX rats, which were also shorter when compared to normal. Cementocytes were incorporated within the matrix in closer proximity to one another than in normal rats, due to the formation of less intervening cemental matrix. Animals treated for 7 days with GH, 2 weeks after hypophysectomy, formed amounts of cellular cementum comparable to normal, although growth in root length did not catch up. Cementocytes, in this group, were measurably further apart due to more intercellular matrix.

Ultrastructurally, hypophysectomy was accompanied by significant reductions in the extent of the rough-surfaced endoplasmic reticulum compartment (rER) in precementoblasts and cementoblasts. However this difference was not found in cementocytes. Growth hormone treatment of HYPOX rats produced ultrastructural changes in precementoblasts and cementoblasts which are usually associated with active protein synthesis and matrix deposition. Qualitative differences were appreciable in the number of nuclear pores and in the pseudopodia of the plasma membrane of these cells, although no significant differences in nuclear or cell area were recorded by morphometry. No measurable differences were found in nuclear parameters with the different treatments although the nuclei of cementocytes were appreciably more heterochromatic and of smaller area than those in cementoblasts. The intracellular compartments of rRER were quantifiably greater than in HYPOX rats. In sum, it appears that precementoblasts and cementoblasts respond to GH by increased protein synthesis and cementum matrix formation, but cementocytes do not, as would be expected by the differential staining for GHrpb of blasts versus cytes (Zhang *et al.*,1992c).

To date the use of specific lectin binding, and IHC for decorin and biglycan has provided only an evaluation of the effect of GH on cementum matrix proteoglycans limited to the small rim of acellular cementum on the rat incisors. However, GalNAc was demonstrated in acellular cementum as was biglycan in normal and GH-treated dwarf animals. Both were absent in untreated dwarf rats. This suggests that cementum matrix proteoglycans are under regulation of GH.

Thus, both of the mesenchymally derived dental hard tissues, dentine and cementum, appear to be under control of GH and IGF in terms of their growth by proliferation and matrix formation.

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