Effect of ascorbic acid deficiency on mouse second molar tooth germs cultivated in vitro

By GORDON E. LEVENSON

From the School of Dental Medicine, University of Pennsylvania

SUMMARY

Mandibular second molar tooth germs from two-day old mice were cultured in vitro, on millipore membranes, for periods of up to 20 days in liquid medium with or without added ascorbic acid. Tooth germs grown in ascorbate medium were characterized by relatively normal growth, differentiation, morphology and histology. Cuspation patterns were maintained. The epithelial root sheath continued to grow along the millipore membrane.

Tooth germs cultured in ascorbate-deficient medium manifested a consistent and striking failure in maintenance of differentiated odontoblastic and ameloblastic tissue with arrest of predentin synthesis, severe structural collapse and reduction in size. Cuspation patterns were lost in scorbutic molars, with sinking of surface layers into pulpal tissue and flattening of the entire organ. This resulted in a lack of recognizable morphology and in severe disorganization of tissues. Only growing areas of the root sheath with associated proliferation of pre-ameloblasts and pre-odontoblasts and adjacent pulpal tissue remained normal and refractory to ascorbate deficiency. Odontoblastic as well as ameloblastic layers were disrupted and cells were dedifferentiated. Newly differentiated odontoblasts became highly vacuolated when they became polarized and started to secrete extracellular matrix.

INTRODUCTION

Mammalian tooth germs have been successfully grown in vitro by many previous workers, and the literature reviewed (Glasstone, 1965; Fisher, 1971). There has been study of a variety of developmental factors including regulation of halved rudiments (Glasstone, 1952), specificity of form (Koch, Koch & Ledbury, 1970; Kollar and Baird, 1970), calcification (Wigglesworth, 1968) and tissue interaction (Slavkin, 1972; Kollar, 1972). In vitro tooth germ culture systems, particularly those utilizing defined media, have interesting potential for experimental manipulation of metabolites in the growth medium. This would permit study of lesions at the cellular level, in relation to abnormal development, as a means of understanding not only pathology but the normal as well. There is a paucity of work in this area.

The clinically manifested 'dental dysplasias' are a group of anatomical abnormalities and structural deficiencies such as insufficient or improperly mineralized dentin and enamel in human teeth which give rise to clinical
problems including enhanced attrition and caries susceptibility. These dysplasias have been related to a variety of causations including febrile diseases, injury, genetic disturbance, vitamin deficiencies and nutritional disorders (Bernier, 1959). These factors have in common that they are all operative during early critical stages of tooth development. The probability of common pathways of action of causative agents has been stressed (Kreshover, 1960).

Ascorbic acid or vitamin C was chosen by the present author as a likely agent for use in developing an in vitro dysplastic dental model system, the main objective of the study reported here. Deficiency of ascorbic acid in animals was shown in 1919 to have widespread effects on tooth development in vivo (Zilva & Wells, 1919). Scorbutil odontoblasts were observed to regress to squamoid form (Wolbach & Howe, 1933). Ameloblasts in developing scorbutic guinea-pig teeth fail in zones with defective odontoblasts (Fullmer, Martin & Burns, 1961). No detailed study has been published on the role of ascorbic acid in tooth development in vitro. Since scorbutic animals suffer widespread damage including inanition and vascular changes, it is difficult with in vivo studies to ascertain whether observed effects are primary. The in vitro system obviates this problem.

The author has previously reported the effects of ascorbic acid deficiency in cell cultures of embryonic chondrocytes in vitro (Levenson, 1969; Levenson, 1970). In these studies ascorbic acid was found to be required for maintenance of reaggregated cell clusters and for expression of unique phenotypes in each of three varieties of embryonic cartilage. Extracellular matrix formation was altered in ascorbic acid-deficient cultures and it was suggested that these effects might involve abnormalities in collagen production. This was subsequently confirmed (Lavietes, 1971). An additional reason for using tooth germs in culture here was to test the role of ascorbic acid on a more complex intact organ system, with differentiating and interacting tissues, which is small enough to permit handling, culturing and experimentation. The tooth germ is ideal for this purpose.

In the present study we were able to show marked alterations in tooth germ development in vitro resulting from ascorbic acid deprivation.

**MATERIALS AND METHODS**

Second molar tooth germs were removed as bilateral pairs from the mandibles of two-day-old Swiss-Webster mice. Tooth germs were placed individually on millipore strips cemented on to stainless steel grids in 18 mm culture dishes and cultured according to the method of Wigglesworth (Wigglesworth, 1968). The dishes containing each pair of tooth germs were kept in a Petri-dish moist chamber. Medium BGJ (Biggers, Gwatkin & Heyner, 1961), compounded in our laboratory, was used throughout and supplemented with 15 % fetal bovine serum (Flow Labs) and 2 % beef embryo extract (Flow Labs). Medium was changed every 48 hours. Ascorbic acid (150 μg/ml) was added as powder to
medium at the time of each medium change. Cultures were maintained at atmospheric pressure under 50% O₂, 45% N₂ and 5% CO₂ in MacIntosh-Fieldes jars at 37 °C. Tooth germs were fixed from the 4th to 20th day of culture. At least two pairs were fixed at the commencement of each experiment just after removal from mandibles. For paraffin embedding, material was routinely fixed in Bouin’s fluid, sectioned at 8 μm and stained with Heidenhain’s iron Hematoxylin, Hematoxylin and Eosin, or Azan. For 1 μm sections, material was fixed in 4% gluteraldehyde in 0.1 M cacodylate buffer, post-fixed in 2% osmic acid in the same buffer, embedded in Epon 812, and sectioned on a Porter-Blum Model MT-1 ultra-microtome. Epon sections were stained with toluidine blue. Material was routinely examined and photographed by light microscopy. A Zeiss polarizing microscope was also used.

RESULTS

A total of 320 pairs of tooth germs were cultured in this study. Seventy-three experiments were performed. Numbers of pairs per experiment depended on litter size, which averaged about ten mice. Second molar tooth germs from two-day-old mice exhibit a definite cuspal pattern (Fig. 1). Differentiated ameloblasts and odontoblasts were seen in cusp tips where a very thin layer of predentin is found (Figs. 1, 1A). This is the stage of initial dentinogenesis (Cohn, 1957). The base of the tooth germ is still ‘open’ since there is as yet no root formation.

(a) 8- to 12-day cultures, ascorbate medium

Tooth germs cultured for 8 days in a medium supplemented with ascorbic acid were consistently characterized by normal morphology and histology (Figs. 2, 2A). Although some flattening was unavoidable in young tooth germs removed from the normal support of contiguous tissues, the cuspal pattern was maintained and tissue relationships were normal. Growth of the epithelial root sheath continued vigorously along the millipore membrane. Instead of the normal growth of the root sheath downward as occurs in vivo, it grew along the millipore membrane from the sides towards the center until it fused centrally, forming a continuous layer at about the eighth day in culture (Fig. 4).

A developmental series of ameloblasts ranging from dividing pre-ameloblasts at the tip of the root sheath, or cervical loop, to fully polarized high columnar ameloblasts was found in this tissue lying just above the millipore membrane (Fig. 4A).

In areas over the membrane where the root sheath had fused, and up the sides, there was a continuous layer of normal ameloblasts over which were differentiated odontoblasts (Fig. 4A). The continual formation of new ameloblasts was associated with differentiation of odontoblasts from adjacent pulpal tissue. The normal sequence of developmental stages of both ameloblasts and odontoblasts
and dentin, progressed from the youngest tissue of the root sheath laterally and upward towards the future occlusal surface.

The stellate reticulum of the enamel organ in some cases remained clearly visible at 8 days while in other cases remnants of it were seen only between cusps (Figs. 2 and 4).

Occlusal and lateral surface ameloblastic epithelium was luxuriant, uniformly thick and fully differentiated, exhibiting tall columnar cells and characteristic polarization (Fig. 2a). A thin layer of enamel matrix appeared in some specimens (Figs. 8, 8A). The odontoblastic layer was intact and well developed with the exception of small zones in some specimens near the cuspal apices where it was irregular, probably due to crowding. The integrity and function of this layer correlated with the amount of dentin matrix deposited. A progressively thickened layer of dentin, thickest at the cusp tips, developed during the 8 days in culture (compare Figs. 1A and 2A). Polarization microscopy indicated that uniformly oriented fibrous material appearing regularly in the dentin layer was birefringent along with masses of birefringent material in the pulp. This dentin and fibrous pulpal matrix stained uniformly blue with Azan stain. These two observations suggest that the fibers are collagenous. The cell population of the pulp proper was homogeneous and the tissue was uniform.

(b) 8- to 12-day culture, ascorbate-deficient medium

Tooth germs cultured for 8 days without added ascorbic acid in the medium underwent a consistent and striking loss of organization; morphology and size were also seriously affected. At 8 days they were an average of 16% smaller than those grown in ascorbate medium when measured at their widest point and were approximately one-half the height of companion tooth germs grown in ascorbate medium. It was impossible to recognize a cuspatation pattern in

---

Figures 1–8
Photographs are from specimens sectioned at 8 μm and stained with Heidenhain's hematoxylin unless otherwise noted.

Figures 1 and 2
Fig. 1. Longitudinal section of normal first molar tooth germ from two-day-old mouse, at time of explantation (see Fig. 1A). (p), pulp; (a), ameloblasts; (s), stellate reticulum. × 250.

Fig. 1A. Enlargement of left cusp portion of Fig. 1. The initial layer of predentin (d) has formed. (o), odontoblasts; (p), pulp; (a), stellate reticulum; (a), ameloblasts. × 800

Fig. 2. Longitudinal section of tooth germ cultivated for 8 days in ascorbate medium. Cuspatation pattern and normal histology have been maintained. The root sheath (s) grew towards the centre from both sides along the millipore membrane (m). (c), cusp tips; (d), dentin layer; (p), pulp. × 200.

Fig. 2A. Enlargement of insert in Fig. 2. (a) ameloblasts; (d), dentin; (o), odontoblasts; (p), pulp. × 600.
Ascorbic acid-deficient tooth germs and often there was an irregular flattening with sinking of the surface layers into pulpal tissue (Fig. 3). Stellate reticulum sloughed off rapidly in many of these cultures. Relatively normal ameloblasts in the occlusal and lateral regions were usually found associated with intact groups of underlying odontoblasts, although the occlusal ameloblast layer was usually deformed (Fig. 3A). Even in tooth germs where gross deformation and disorganization of tissue occurred, the growing portion of root sheaths and associated pulpal tissue remained normal and completely refractory to ascorbate deficiency (Fig. 3). Despite this pathology, growth of the root sheaths, division of cells in the contiguous pulpal tissue and differentiation of new odontoblasts continued normally as in ascorbate medium.

Cultures were treated with colcemid (Ciba-Geigy, 0.05% for 3 h) prior to fixation on the 8th day, and comparison was made in alternate 8 μm serial sections of the numbers of dividing cells in the root sheaths, of 30 paired cultures from five experiments. No significant differences were found between tooth germs grown in media with or without ascorbic acid. Actively dividing pre-ameloblasts, pre-odontoblasts and associated growing pulpal tissue at the base of the tooth germ continued to proliferate in vitro as in vivo.

Changes seen at 12 days were intermediate between those described for ascorbate-deficient cultures at 8 days and 20 days. By the twelfth day in culture, dentin thickness was greatly diminished in comparison with ascorbic acid-treated cultures and did not exceed the dentin thickness deposited by 4- to 5-day ascorbate-treated cultures. These effects may be seen at 20 days in culture by comparing Figs. 6, 6A and 7, 7A. Deposition of dentin had become completely arrested in ascorbate-deficient cultures. In 1 μm sections the dentin appeared

---

**Figures 3 and 4**

Fig. 3. Longitudinal section of contralateral tooth germ to that in Fig. 2, grown for 8 days in ascorbate-deficient medium. Morphology is severely disorganized, with deterioration into viable but gnarled masses of tissue. Growing cervical loop areas of root sheath (s) remained normal. (p), pulp. × 200.

Fig. 3A. Enlargement of insert in Fig. 3. Ameloblasts (a) have lost their high columnar and polarized form and have formed a knot (within arrows). Odontoblasts were no longer distinguishable and production of dentin matrix did not occur. (p), pulp. × 600.

Fig. 4. Cross-section of tooth germ cultured for 8 days in ascorbate medium. Dentin in region of cusp tips is beginning to calcify (c). (a) ameloblasts; (d), predentin; and (o), continuous odontoblast layer. The root sheath has grown together centrally to form a continuous layer over the millipore membrane (m). 1 μm section, Epon embedded. × 200.

Fig. 4A. Enlargement of lower insert in Fig. 4. Recently differentiated odontoblast (o), ameloblasts (a) and new predentin (d). A thin, characteristic layer of fibroblasts (f) is seen between ameloblasts and millipore membrane. × 500.

Fig. 4B. Enlargement of upper insert in Fig. 4. Dentin matrix (d) contains odontoblastic processes. (o), odontoblast layer; (a) ameloblasts. × 825.
thin, irregular, and with a crinkled texture and discontinuous odontoblast layer (compare Figs. 5 A and 4B). The odontoblast layer, except in the root sheath area over the millipore membrane, was discontinuous or severely fragmented, and depolarized cells derived from the degenerating odontoblast layer were frequently observed penetrating well into the pulp proper although still attached to odontoblastic processes which entered dentinal tubules. In some areas no distinguishable odontoblasts were seen in any form. Ameloblasts often deteriorated into squamoid or other forms. Small spaces appeared in the central pulp although there was never any cellular evidence of necrosis. Azan staining resulted in an overall light blue coloration of pulp, with no large blue-staining fibers as seen in ascorbic acid-treated specimens. A strong difference in toluidine-blue staining intensity in the pulp was also noted in Epon sections (compare Figs. 4 and 5). Similarly, dentin and pulp of ascorbate-deficient cultures exhibited no birefringence when examined by polarizing microscopy and when compared with controls. A striking and consistent feature of ascorbate-deficient cultures was the appearance of large vacuoles in odontoblasts only in the zone where they had recently differentiated (Fig. 5B).

(c) 20 days in culture, ascorbic-acid medium

At 20 days in culture, tooth germs grown with added ascorbic acid had not grown in size when compared with those at 12 days. A definite cuspation pattern was retained although frequently somewhat distorted (Fig. 6). A well-differentiated ameloblast layer was always observed laterally and over the millipore membrane, although in some cases occlusal ameloblasts were low cuboidal or squamoid. Similarly, the occlusal odontoblast layer was not always intact except in areas where dentin matrix was still being formed; it was always normal on the sides and over the millipore membrane. Pulpal tissue was the same as at 12 days.

Figures 5 and 6

Fig. 5. Cross-section of contralateral tooth germ to that in Fig. 4, grown for 6 days in ascorbate-deficient medium. Dentin appears as two layers, dark and light staining (see Fig. 7a). (a), ameloblasts; (p), pulp; (d), dentin. × 200.

Fig. 5 A. Enlargement of upper insert in Fig. 5. Dentin matrix (d) is irregular and lacks tubular structure. (a), ameloblasts; (o), odontoblasts. × 600.

Fig. 5B. Enlargement of lower insert in Fig. 5. Recently differentiated odontoblasts (o) exhibit characteristically large vacuoles (arrows) which develop characteristically in ascorbate-deficient cultures (see text). (a), ameloblasts; (p), pulp. × 600.

Fig. 6. Longitudinal section of tooth germ cultured for 20 days in ascorbate medium. Although somewhat flattened, the cuspation pattern was retained. A layer of dentin was formed in vitro (d) and ameloblasts were well maintained (see Fig. 3 A). (d), dentin; (m), millipore; (p), pulp. × 200.

Fig. 6A. Enlargement of insert in Fig. 6. (a), ameloblasts; (p), odontoblasts; (d), dentin; (s), space due to fixation shrinkage. × 600.
Tooth germs cultured without added ascorbic acid were severely flattened, with no recognizable morphology. There was little or no cuspal pattern (Fig. 7). Tissue was arranged in lumps and large whorls, often containing tumour-like masses composed of remnants of ameloblastic tissue (Fig. 7, 7A). The ameloblastic layer in these usually appeared as a row of low columnar or cuboidal cells on the occlusal surface and over the millipore membrane. No recognizable odontoblasts were seen except in sparse patches over the millipore-associated ameloblasts. The cellularity of the pulpal tissue per unit area in sections appeared to be greater than in ascorbate cultures. No predentin was seen in any of these cultures, although some had a very thin deformed dentin layer resembling that at the stage of explantation.

The entire structure tended to collapse except for root sheath areas. This was not due to closer proximity of this area to the millipore material since results were the same in tooth germs grown inverted on millipore membranes.

**DISCUSSION**

In ascorbate-deficient medium, progressive and widespread changes were observed. There was a loss of normal morphology and integrity of differentiated odontoblast and ameloblast layers in scorbutic tooth germs. Production of predentin ceased. However, proliferation of cells giving rise to new ameloblasts and odontoblasts continued normally in the growing cervical loop region. Differentiation of new odontoblasts and ameloblasts progressed normally. Odontoblasts, however, when they became polarized, and at the stage of initial predentin secretion, became highly vacuolated. This was never observed in the pre-odontoblasts or in older odontoblasts. The vacuolation suggests the occurrence of some disturbance, possibly metabolic, in newly differentiated odontoblasts in the absence of ascorbic acid, since this phenomenon was never

---

**Figures 7 and 8**

Fig. 7. Longitudinal section of contralateral tooth germ to that in Fig. 6, grown for 20 days in ascorbate-deficient medium. Collapse of entire tooth germ structure has occurred, with no recognizable cuspal pattern and lack of normal tissue relationships. Gnarled mass of ameloblasts within the insert. (p), pulp; (a) ameloblasts; (m), millipore. × 200.

Fig. 7A. Enlargement of insert in Fig. 7. Degenerating and gnarled ameloblast layer (a); thin remnant of dentin matrix (d). × 600.

Fig. 8. Longitudinal section of tooth germ grown in ascorbic-acid medium for 12 days. A thickened layer of enamel matrix (e) has been deposited. (d), dentin; (p), pulp. Fixed in Zenker's fluid. Azan stain. × 300.

Fig. 8A. Enlargement of insert in Fig. 8. (a) ameloblasts; (e), enamel; (d), dentin; (p), pulp. × 825.
observed in ascorbate-supplemented cultures. The apparent increase in cellularity of pulpal tissue in scorbutic cultures most likely reflects a decrease in extracellular matrix rather than an increase of cell proliferation.

The major results of ascorbic-acid deficiency in this system are failure to maintain differentiated odontoblasts and ameloblasts, appearance of disorganized dentin matrix, cessation of dentin production and overall structural collapse.

What primary lesion could result in this experimentally produced pathology in vitro? The bulk of protein made by the dental pulp and dentin is collagen (Eastoe, 1967). It is now accepted that ascorbate is a cofactor for enzymic hydroxylation of proline and lysine in collagen synthesis (Bornstein, 1974). Stability of the collagen molecule at body temperature has recently been shown to depend on its hydroxyproline content (Jiminez, Harsch & Rosenbloom, 1973). Ascorbate deprivation could therefore result in insufficient or abnormal collagen synthesis at incubation temperature, so that tissues such as dentin and pulp would be deprived of their major structural protein. There may be other effects on the cells, due to altered mechanisms of synthesis or altered or deficient extracellular matrix. Extracellular collagen in addition to mucopolysaccharides with which it interacts has been implicated in morphogenesis and maintenance of the differentiated state (Trelstad, 1973). The author reported previously that maintenance of unique phenotypes in cell cultures of three varieties of embryonic chondrocytes required ascorbic acid in the medium. Without this vitamin cartilage tissue formed de novo by reaggregated cells was not maintained and matrix production ceased. I suggested the possible importance of extracellular matrix components, particularly collagen, in these effects (Levenson, 1970). Dependence of cartilage matrix synthesis on ascorbic acid in vitro was then demonstrated; it was suggested that a lack of collagen resulted in failure to retain glycosaminoglycans in insoluble form in the matrix (Lavietes, 1971).

The collapse of the entire tooth-germ structure in ascorbate-deficient medium as reported here, is most likely related to a failure in the maintenance of pulpal tissue fabric owing to deficient or aberrant collagen production. It seems reasonable to visualize surface layers of tooth germs, while undergoing histogenesis and morphogenesis of cusps, as being physically supported at early stages by the underlying bulky embryonic pulp. As dentin and enamel matrix are developed, and particularly as they calcify, they would constitute an architectural shell which would provide additional support for developing tissues and maintain form and contour while root formation proceeded. The flattening and deterioration of the organized mass of tooth germs observed here could be due to structural failure, resulting from abnormal matrix synthesis in both pulp and dentin. Although we do not yet know whether ameloblast deterioration in this lesion is a primary or secondary effect, dependence of ameloblast maintenance and integrity on subjacent odontoblasts has been noted by Fullmer et al. (1961).
Ascorbic acid-deficient tooth germs

The technical assistance of T. Keller, R. Karp, K. Kobos and H. Price is gratefully acknowledged. This work was supported by U.S.P.H.S. grant DE-02623 to the Center for Oral Health Research, University of Pennsylvania, Philadelphia, Pa 19174.

REFERENCES


(Received 21 November 1975; revised 1 April 1976)