Tunicamycin inhibits mouse tooth morphogenesis and odontoblast differentiation in vitro

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SUMMARY

Tunicamycin (TM), an antibiotic that selectively inhibits dolichol-mediated protein glycosylation, inhibited morphogenesis and differentiation of odontoblasts in the molar tooth germ in vitro. These effects of TM are reversible and dose-dependent, and in advanced teeth the effect of TM was not complete unless the basement membrane was removed prior to culture. TM did not prevent secretion of predentin or enamel when added to the cultures after initiation of predentin secretion. TM dramatically inhibited protein glycosylation and the accumulation of labeled proteoglycans and glycoproteins in the basement membrane. Our previous studies indicated that odontoblast differentiation is triggered by an interaction between the basement membrane and mesenchymal cells. We suggest that TM inhibits odontoblast differentiation by causing alterations in the basement membrane which prevent the necessary cell–matrix interaction required for odontoblast differentiation.

INTRODUCTION

The tooth bud is initiated when the oral epithelium grows into the underlying mesenchyme. Tooth development reaches the bell stage when the epithelial bud invaginates at its mesenchymal aspect and grows to surround a condensation of mesenchymal cells, known as the dental papilla. At this time, cuspal morphogenesis begins and epithelial-mesenchymal cell differentiation and matrix secretion take place. The mesenchymal cells first differentiate into odontoblasts and secrete predentin. Subsequently, the epithelial cells apposing the predentin polarize into ameloblasts and deposit the organic matrix of enamel. Interaction between the epithelial and mesenchymal components of the tooth germ appears to be a prerequisite for morphogenesis and differentiation since isolated tooth epithelium or mesenchyme is not able to form dental structures (Koch, 1967; Kollar & Baird, 1969).

The events that occur during these tissue interactions are not known, but some information has been obtained from various experimental studies. Culture of heterochronal associations of dental epithelium and mesenchyme has shown...
that these tissues influence the mitotic activity of each other (Ruch, Karcher-Djuricic & Thiebold, 1976). Transfilter studies have indicated that the mesenchymal cells need to come into close contact with the basement membrane before they differentiate into odontoblasts (Thesleff, Lehtonen & Saxén, 1978). The importance of the extracellular matrix has also been demonstrated in studies in which inhibition of collagen synthesis or processing inhibited tooth morphogenesis (Ruch, Fabre, Karcher-Djuricic & Staubli, 1974; Galbraith & Kollar, 1974; Hetem, Kollar, Cutler & Yager, 1975). Diazo-oxo-norleucine (DON) is a glutamine analogue which interferes with glycoconjugate synthesis and glycosylation and inhibits rodent tooth morphogenesis and odontoblast differentiation in vitro (Hurmerinta, Thesleff & Saxén, 1979). It was suggested that DON exerted its effect on the developing tooth by preventing the interaction between the mesenchymal cells and the basement membrane. However, in addition to its inhibitory effect on glycoconjugate synthesis, DON also affects purine biosynthesis (Buchanan, 1973).

Tunicamycin is an antibiotic that selectively interferes with glycoprotein biosynthesis by inhibiting the formation of dolichol-bound N-acetylglucosamine derivatives (Takatsuki, Kohno & Tamura, 1975). Consequently, the glycosylation of proteins at asparagine residues is prevented (Tkacza & Lampen, 1975; Olden, Pratt & Yamada, 1978). In the present study we have examined the effect of tunicamycin on tooth morphogenesis. Our results show that tunicamycin inhibits the morphogenesis of cultured mouse tooth germs, and it selectively inhibits the differentiation of odontoblasts if present at the early stages of development. At later stages, differentiation is inhibited only if the basement membrane is removed prior to cultivation.

**MATERIAL AND METHODS**

Tooth buds were dissected from mouse fetuses of days 14–17 of gestation. Male and female Swiss Webster (NIH) mice were allowed to mate overnight and the detection of a vaginal plug was considered day 0 of gestation. The lower jaw of each embryo was removed, submerged in phosphate-buffered saline (PBS, pH 7.4), and the first mandibular molars, containing a piece of overlying oral epithelium, were dissected out. In order to remove the basement membrane, the molars of day-16 fetuses were treated with 2.25% trypsin-0.75% pancreatin for 15 min at 4 °C. The tissues were either placed directly in culture after enzyme treatment or were first incubated for 30 min in culture medium at 22 °C. In the latter case, the epithelial and mesenchymal components of the tooth bud were then mechanically separated, and cleaned of adherent tissues. The mesenchyme was first placed on the Millipore filter followed by the inner enamel epithelium which was placed in direct contact with the mesenchyme.

A Trowell-type organ culture system was used, with the explants supported by a Millipore filter (25 μm thick, 0.45 μm porosity) on a metal grid. The
medium consisted of BGJb medium (GIBCO) supplemented with 100 i.u./ml penicillin, 100 µg/ml streptomycin, 0-25 µg/ml Fungizone, 20% horse serum (GIBCO), 10% chick embryo extract, and 150 µg/ml ascorbic acid. The culture dishes were kept in a humidified incubator at 37 °C in an atmosphere of 5% CO₂ in air. The explants were cultured for 2–14 days, and the medium was changed every other day. The tissues were fixed in Carnoy’s or Zenker’s solutions, embedded in Paraplast and serially sectioned at 6 µm. Deparaffinized sections were stained with periodic acid-Schiff reagent (PAS) or Mallory’s phosphotungstic acid-hematoxylin.

The incorporation of labelled precursors into glycoproteins and proteoglycans was examined by autoradiography. During the third day of culture, the explants were exposed for 6 h to media containing 50 µCi/ml of either D-[2-³H(N)]-mannose (specific activity 18 µCi/mmol), L-[6-³H]-fucose (specific activity 13-1 Ci/mmol), or [³⁵S] sodium sulfate, all from New England Nuclear. Afterwards the explants were washed three times in PBS containing 5 mM unlabelled precursor. Explants were fixed in Carnoy’s solution, embedded in Paraplast and sectioned at 6 µm. The deparaffinized and hydrated sections were dipped in nuclear track emulsion NTB-2 (Kodak). The slides were exposed at 4 °C for 1–4 weeks, developed, and stained with PAS.

Tunicamycin was a gift from Dr Gakuzo Tamura via the Drug Evaluation Branch of the National Cancer Institute. It was dissolved in DMSO (dimethylsulfoxide) and stored at −70 °C and used in concentrations from 0-01 µg/ml to 2 µg/ml. In most experiments, TM at a concentration of 0-15 µg/ml was used, and an equivalent volume of DMSO was included in control medium.

RESULTS

Cultures of whole tooth rudiments

At day 14 the molar tooth bud is in the cap stage of development, and the epithelial enamel organ partially surrounds the dental papilla mesenchyme. When cultured in control medium, the tooth germs reached the bell stage by 4 days of culture, and by 10 days the differentiated odontoblasts had secreted predentin and the ameloblasts had polarized (Fig. 1A). In the presence of TM (0-15 µg/ml), the developmental stage of the tooth germs was the same after 4 days of culture as at the onset of cultivation (Fig. 1B); after 10 days the tooth rudiment had regressed and the epithelia were heavily keratinized.

In the day-15 fetus, the molar has reached the bell stage of development and the onset of cuspal morphogenesis is already evident. TM prevented further cuspal morphogenesis when added to these cultured rudiments. Differentiation of odontoblasts, however, was not completely prevented and, in three of nine TM-treated tooth germs, some predentin was secreted by polarized odontoblasts in the cuspal area (Fig. 1C, D).

Cuspal morphogenesis is more advanced in the day-16 and day-17 molars,
Fig. 1. Photomicrographs illustrating the effect of tunicamycin (TM, 0.15 μg/ml) on the development of mouse molar tooth germs in culture. Periodic acid–Schiff stain.

(A) Tooth germ of a day-14 fetus cultured for 10 days in control medium. Cuspal morphogenesis is advanced; the differentiated odontoblasts (O) have secreted predentin (PD) and the ameloblasts (A) have polarized.

(B) A day-14 tooth germ cultured for 4 days in the presence of TM. Morphogenesis has been inhibited, and the mesenchymal cells (M) have not differentiated. Dental epithelium (E).

(C) A day-15 tooth germ cultured for 7 days in control medium. Well-developed cusps are seen; differentiated odontoblasts (O) have secreted predentin (PD).

(D) A day-15 tooth germ cultured for 7 days in the presence of TM. Cuspal morphogenesis has been inhibited. This is one of the three explants where the odontoblasts at the cusps differentiated and secreted predentin (PD). In the remaining six explants no predentin was seen.
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but predentin secretion is not yet observed. In control cultures of day-16 and -17 molars, well-developed cusps were seen after 7 days of cultivation. Differentiated odontoblasts which had secreted predentin were observed. Ameloblasts had polarized (Fig. 2A) and, in a few explants, secreted enamel. In the presence of TM at 0.15 μg/ml, cuspal development in the tooth germs remained at the same stage as it was at the onset of culture; no predentin was secreted in four out of six day-16 molars cultured for 7 days (Fig. 2B). In day-17 molars, TM did not prevent predentin secretion, but cuspal morphogenesis was inhibited (not shown).

The effect of TM was reversible. In experiments where the day-16 tooth germs were transferred to control medium after 4 days of culture in the presence of TM (0.15 μg/ml), predentin secretion and ameloblast polarization were observed after 10 days of culture (Fig. 2C). Under these conditions, the explants were smaller than the controls, but cuspal morphogenesis was well advanced.

TM did not prevent predentin secretion, ameloblast polarization or enamel secretion if the day-16 tooth germs were cultured for 4 days in control medium prior to addition of TM. After 4 days in control medium, the polarized odontoblasts had secreted the first layer of predentin, but ameloblasts were not polarized. During the following 10 days in the presence of TM, additional predentin was secreted, and by the end of this period the ameloblasts had polarized and begun to secrete enamel (Fig. 2D).

Cultures of enzyme-treated tooth germs

Incubation of tooth germs in the 2.25% trypsin–0.75% pancreatin solution results in the removal of the basement membrane (Thesleff et al. 1978). The enzyme-treated molars of day-16 fetuses, when grown in control medium, developed in the same manner as control molars. After 6 days in culture, well-developed cusps were observed, predentin had been secreted by the differentiated odontoblasts, and the ameloblasts had polarized (Fig. 2E). TM prevented cuspal morphogenesis, differentiation of the mesenchymal cells into odontoblasts and subsequent predentin secretion and ameloblast polarization in all 12 explants (Fig. 2F). No pyknotic nuclei indicative of cell death were observed in these cultures, and the effect of TM was reversible. The explants transferred to control medium after 4 days of culture in the presence of TM (0.15 μg/ml) recovered in the same manner as non-enzymically-treated tooth germs (see above).

Cultures of recombined epithelium and mesenchyme

When dental epithelium and mesenchyme of day-16 molars were enzymically separated and recombined in culture, odontoblasts and ameloblasts differentiated in a manner similar to that of cultures of whole tooth germs. After 2 days of culture in control medium, the mesenchymal cells had aligned under the enamel epithelium and a distinct PAS-positive basement membrane was
observed (Fig. 3A). After 7 days predentin was deposited and the ameloblasts had polarized (Fig. 3C). The extent of the normal cusp pattern could not be assessed in these explants (compare Fig. 1C with Fig. 3A). TM prevented differentiation of odontoblasts, and the effect was dose-dependent: 0.01 μg/ml of TM had no effect, and predentin was secreted in all five explants after 6 days of culture; 0.05 μg/ml of TM prevented odontoblast differentiation in three of five explants, whereas 0.1 and 0.2 μg/ml of TM prevented differentiation in all 19 explants. TM at 2 μg/ml was toxic and therefore the concentration of 0.15 μg/ml of TM was used in all subsequent studies. After 2 days of culture in the presence of TM, no alignment of the mesenchymal cells was observed and the basement membrane appeared irregular and was not uniformly PAS-positive as in the controls (Figs 3A and B). After 7 days the TM-treated explants looked similar to those after 2 days of culture, and although these explants were smaller than the controls, there was no obvious cell death (Figs 3C and D).

The effect of TM was reversible when the explants were transferred to control medium after 3 days of culture (Fig. 3E). However, if they were cultured with TM for 4 days, only one out of four explants recovered. None of the seven explants recovered when cultured for 7 days in the presence of TM prior to transfer into control medium. TM did not prevent secretion of predentin, polarization of ameloblasts or enamel secretion if the explants were first grown in control medium for 4 days. At this time the odontoblasts had already begun to secrete predentin. After 7 days of culture in the presence of TM more

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**Figure 2**

Photomicrographs illustrating the effect of tunicamycin (TM, 0.15 μg/ml) on the development of molar tooth germs from day-16 mouse fetuses. Mallory's phosphotungstic acid–hematoxylin stain.

(A) After 7 days in control medium well-developed cusps are seen, and odontoblasts (O) have secreted predentin (PD).

(B) After 7 days in the presence of TM, cuspal morphogenesis is inhibited. This is one of the four explants where no predentin was detected. In the remaining two explants some predentin had been secreted in the cuspal area. Dental epithelium (E), dental mesenchyme (M).

(C) A tooth germ cultured first in the presence of TM for 4 days and then for 10 days in control medium. Predentin has been secreted and cuspal morphogenesis has advanced, indicating partial recovery from TM. However, the explant is smaller than those cultivated in control medium for 14 days.

(D) A tooth germ cultured for 4 days in control medium and then for 10 days in the presence of TM. TM has not prevented secretion of predentin (PD) and enamel matrix (EM).

(E) A tooth germ incubated in 2.25% trypsin–0.75% pancreatin to remove the basement membrane, and then cultured for 7 days in control medium. Well-developed cusps are seen; odontoblasts (O) have secreted predentin (PD).

(F) A tooth germ similar to the one in Fig. 2E, but cultured in the presence of TM. Cuspal morphogenesis has been prevented; mesenchymal cells (M) have not differentiated into odontoblasts.
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predentin had been secreted and the ameloblasts had polarized (Fig. 3F) and occasionally secreted enamel matrix.

**Autoradiographic examination of incorporation of radioactive precursors**

The effect of TM on the incorporation of $[^3]H$mannose and $[^3]H$fucose, precursors of glycoproteins, and $[^35]S$ sulfate, a precursor of sulfated glycosaminoglycans, was examined. Explants of enzyme-treated whole tooth germs of day-16 fetuses were incubated for 6 h with the radioactive precursors on the third day of culture. The incorporation of $[^3]H$fucose was particularly high at the basement membrane region in the control explants (Fig. 4A). In the TM-treated explants, very little incorporation was seen in the basement membrane area, although no clear decrease in the overall incorporation between the controls and the TM-treated explants was detected (Fig. 4B). TM significantly decreased the incorporation of $[^3]H$mannose in the mesenchymal and epithelial tissues (Fig. 4C, D). A deposition of silver grains from $[^35]S$sulfate was also seen in control cultures in the basement membrane area. TM substantially decreased the number of grains observed in this region, but had no effect on the overall incorporation (Fig. 4E, F). It is evident (Fig. 4) that the cell density in the TM-treated explants is higher than that observed in the controls, which suggests that TM reduced the amount of extracellular matrix material in the mesenchymal tissue.

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**Figure 3**

Photomicrographs illustrating the effect of TM (0.15 μg/ml) on explants of dental epithelium and mesenchyme that were enzymically separated and recombined for culture. Periodic acid–Schiff stain (PAS).

(A) An explant grown for 2 days in control medium. A distinct PAS-positive basement membrane is present between the epithelium (E) and the mesenchyme (M), and mesenchymal cells have begun to be aligned under the basement membrane.

(B) After 2 days of culture in the presence of TM, the epithelial-mesenchymal interface is irregular and the basement membrane (arrows) is not uniformly PAS-positive as in the controls (Fig. 3A).

(C) An explant grown for 7 days in control medium. Odontoblasts (O) have differentiated and secreted predentin (PD), and the ameloblasts (A) are polarizing.

(D) In the presence of TM differentiation of the mesenchymal (M) cells into odontoblasts has been prevented. The explant is similar to those after 2 days of culture (Fig. 3B).

(E) An explant grown first for 3 days in the presence of TM and then for 7 days in control medium. Predentin (PD) has been secreted by the differentiated odontoblasts (O), suggesting recovery from TM treatment.

(F) An explant grown first in control medium for 4 days and then in the presence of TM for 7 days. TM has not prevented the secretion of predentin (PD) or ameloblast (A) polarization.
DISCUSSION

Tunicamycin (TM) has been shown to inhibit differentiation in a number of developing systems. It blocks sea-urchin embryogenesis at gastrulation (Schneider, Nguyen & Lennarz, 1978; Heifetz & Lennarz, 1979), and interferes with compaction and blastocyst formation in early mouse embryos (Surani, 1979; Atienza-Samols, Pine & Sherman, 1980). It also inhibits kidney tubulogenesis (Ekblom et al. 1979) and myoblast fusion (Olden, Law, Hunter & Parent, 1980). The biochemical effect of TM is specific and has been well established. It inhibits the formation of dolichol-diphospho-N-acetylgalactosamine thereby preventing the glycosylation of asparagine residues of glycoproteins (Takatsuki et al. 1975; Tkacz & Lampen, 1975). In all of the studies where TM prevented embryonic differentiation, its action appeared to result from alterations in cell-surface glycoproteins.

Our results show that TM inhibited morphogenesis in cultured mouse molar tooth germs. TM also prevented differentiation of odontoblasts with subsequent inhibition of predentin secretion, ameloblast polarization and enamel secretion. This effect of TM was observed in tooth germs from early developmental stages or from later stages providing the dental basement membrane was enzymically removed before cultivation. Furthermore, our results indicate that TM did not prevent deposition of predentin, ameloblast polarization or enamel secretion if it was introduced after the time that odontoblasts had begun predentin secretion.

In the TM-treated tooth germs examined in this study, cuspal morphogenesis was inhibited and the tooth germs were smaller than the controls. It is likely that the reduction in size was due to a decrease in secretion of extracellular
matrix material and to a small decrease in cell proliferation. Whether the effect of TM on cell proliferation was direct or indirect cannot be determined from our results. In the chick embryo fibroblast, TM has been reported to have only limited effects on cell proliferation (Olden et al. 1978). In isolated dental mesenchyme in culture the rate of proliferation is minimal, and it has been shown that mitosis in this tissue is dependent upon the presence of dental epithelium (Ruch et al. 1976). Therefore, we suggest that TM decreases proliferation in the dental mesenchyme by impairing the interaction between the epithelium and mesenchyme rather than by having a direct effect on mesenchymal tissue.

TM inhibited odontoblast differentiation in all cultured tooth germs from day-14 mouse fetuses but only in 70% of the day-15 and -16 tooth germs. In day-17 molars, TM did not prevent odontoblast differentiation. However, TM inhibited odontoblast differentiation in the older tooth germs if the basement membrane was enzymically removed prior to culture. It was shown earlier that the restoration of the basement membrane in such explants is a prerequisite for odontoblast differentiation (Thesleff et al. 1978; Karcher-Djuricic et al. 1978). One possible mechanism by which the basement membrane triggers odontoblast differentiation is through stimulation of proliferation in the pre-odontoblastic cells. It has been suggested that before their terminal differentiation, these mesenchymal cells must go through a specific number of cell cycles (Ruch, Karcher-Djuricic & Thiebold, 1976) as has been suggested for other embryonic cell types (Holtzer, 1970). We have proposed that an additional function for the basement membrane is to trigger odontoblast differentiation by serving as a substratum for the alignment and polarization of the pre-odontoblastic cells (Thesleff, 1978; Thesleff, Stenman, Vaheri & Timpl, 1979). TM may therefore prevent odontoblast differentiation by altering the composition of the basement membrane and/or the mesenchymal cell surface, thereby preventing the necessary cell–matrix interaction.

An examination of incorporation of radioactive precursors into control and TM-treated tooth germs indicated that TM had similar biochemical effects as reported earlier for other systems (Olden et al. 1978; Surani, 1979). Incorporation of 2-[\(^{3}\text{H}\)]mannose, which is a specific precursor of glycoprotein biosynthesis, was dramatically reduced by TM. Furthermore, in the basement membrane of the control explants, particularly high incorporation of [\(^{5}\text{H}\)]fucose and [\(^{35}\text{S}\)]sulfate was observed, and this was dramatically reduced in the TM-treated tooth germs. This suggests that the basement membrane of the TM-treated tooth germs contained lesser amounts of glycoproteins and sulfated glycosaminoglycans than did controls.

Fibronectin, which is a major glycoprotein of the extracellular matrix and cell surface, is present in dental basement membrane in large amounts at the time of odontoblast differentiation (Thesleff et al. 1979). One possible function of fibronectin is to mediate mesenchymal cell alignment and polarization. TM
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prevents glycosylation of fibronectin in various cells in culture (Olden et al. 1978; Duksin & Bornstein, 1977), and it has been shown that proteolytic degradation of the unglycosylated fibronectin results in decreased amounts of fibronectin at the cell surface and medium from cultured cells (Olden et al. 1978). It is therefore possible that the TM-induced inhibition of odontoblast differentiation observed in the present study resulted from reduced amounts of fibronectin in the basement membrane and at the cell surfaces. All of these alterations in the basement membrane presumably result in inhibition of the necessary cell–matrix interaction between the mesenchymal cells and the basement membrane.

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REFERENCES


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