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Induction of cleft lip in cultured rat embryos by localized administration of tunicamycin

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SUMMARY

Whole-embryo culture techniques have advanced to the point where the study of normal and abnormal primary palate development *in vitro* is possible. The purpose of this study was to ascertain whether localized administration of tunicamycin (TM) an inhibitor of protein glycosylation, into the region of the developing primary palate would induce cleft lip in culture. Rat embryos were explanted on late day 11 of gestation and cultured with open yolk sacs for 40 h. TM was administered by implanting a sterile eyelash soaked in TM into the nasal placode region. The contralateral placode was used as the control by implanting an eyelash lacking TM. Under these conditions, TM-treated placodes were found to develop cleft lip in 14 out of 15 embryos compared to 0 for the controls. These experiments demonstrate that localized administration of TM results in cleft lip formation in whole embryo culture. The technique of localized administration of drugs and teratogens in whole embryo culture should prove useful for similar studies on embryonic development.

INTRODUCTION

Formation of the primary palate in the rat embryo takes place from late day 11 through day 13 of gestation. Invagination of the nasal placode initiates formation of the lateral and medial processes. These opposing nasal processes make contact and fuse from posterior to anterior (Trasler, 1968; Lejour, 1970; Pourtois, 1972; Gaare & Langman, 1977). Fusion of these processes along with the maxillary processes gives rise to a number of midline craniofacial structures including the primary palate. In recent studies on the fusion of the facial processes, it has been shown that the epithelium of the medial and lateral nasal processes secretes a carbohydrate-rich surface coat which is presumed to be necessary for adhesion of the nasal processes (Smuts, 1977; Gaare & Langman, 1977; Figueroa & Pratt, 1979). It has previously been shown that the presumptive

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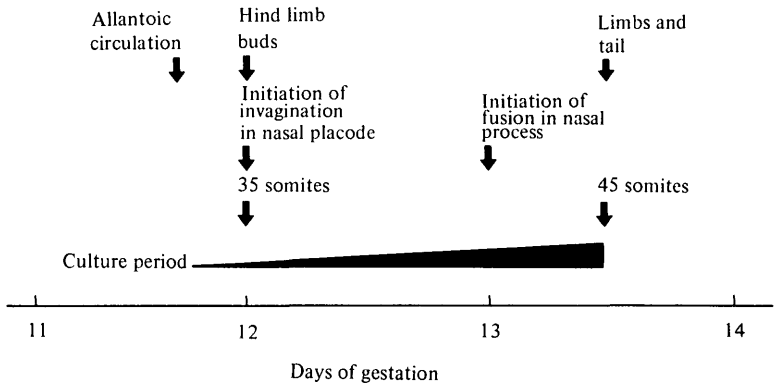

Fig. 1. Period of primary palate formation of rat embryos *in vitro*.

fusion epithelium of the secondary palatal shelves secretes a carbohydrate-rich surface coat (Pratt & Hassell, 1975; Souchon, 1954) which is necessary for the adhesion of the palatal shelves *in vitro* (Greene & Pratt, 1977).

Complete formation of the rodent primary palate is now obtained in culture using rat embryos from late day 11 through day 13 of gestation (Fig. 1) due to improvements in culture techniques and media (reviewed by New, 1967; New & Coppola, 1970; New, 1978). It would be of interest to be able to specifically alter facial development with teratogens in culture. The purpose of this study was to develop a suitable technique for localized administration of drugs to whole embryos in culture. We used the antibiotic, tunicamycin, which specifically inhibits the glycosylation of asparagine residues. The lack of these carbohydrate units results in abnormal secretion or function of many glycoproteins such as fibronectin (Olden, Pratt & Yamada, 1978).

MATERIALS AND METHODS

Source of embryos

Male and female rats of the Sprague-Dawley strain, weighing approximately 250 g, were placed together overnight. The morning that vaginal smears were found to be sperm positive was considered as day 0 of gestation.

Explantation and culture of embryos

On late day 11 of gestation, the uterus was removed and maintained in phosphate-buffered saline (PBS) at 4 °C. All procedures were carried out under sterile conditions. The uterus was opened with fine forceps and each conceptus was isolated with dissecting scissors. The decidua was then removed with watchmaker's forceps and Reichert's membrane was opened, exposing the yolk sac (New, 1967). The yolk sac was opened using techniques similar to those described by Cockroft (1973). The embryo, containing four to six tail somites, was then

transferred to culture vials (2·5 cm diameter by 6 cm length) containing 5 ml of media. The media was composed of 70% Waymouth's (752/1) (Grand Island Biological Co., N.Y.) and 30% heat-inactivated rat serum plus both penicillin and streptomycin at final concentrations of 50 units/ml and 50 μg/ml, respectively. The culture vials were gassed for 20 sec at 0 and 24 h with 95% O_2 and 5% CO_2 and rotated at 8 RPM at 37 °C.

Evaluation of growth and differentiation of cultured embryos

Embryos were cultured for 40 h and then dissected free of membranes and placenta. Somite number was determined, using the somite caudal to the hind limb bud as number 32 (Witschi, 1962). The protein content of each embryo was measured by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). Cultured embryos were compared with embryos from same mother maintained at 4 °C and with embryos from another mother taken 16 h after the time of initial explanting for culture in order to have equal numbers of somites.

Localized administration of tunicamycin

A sterile human eyelash (Eto, Figueroa, Tamura & Pratt, 1978) was dipped into a solution of tunicamycin of 0·5 mg/ml of methanol for 30 min at 22 °C, dried at 4 °C for 12 h and then implanted in the region of the slightly indented nasal placode. The eyelash (about 0·2 mm length) was implanted between the presumptive medial and lateral processes. The contralateral placode of each embryo was used as control by implanting an eyelash not containing TM, but treated in methanol and dried as previously described.

Bioassay of the amount of tunicamycin bound to each eyelash

Chick embryo fibroblasts were grown as described by Olden, Pratt & Yamada (1978). Cells were grown in 35 mm culture dishes and either treated with TM at 0·05 μg/ml or with TM-treated eyelash which was placed directly in the culture dish. The cells were cultured in the presence of the TM for 24 h at which time 5 μCi/ml of ^{3}H-(2)-mannose (New England Nuclear) was added to the culture medium. After 6 h, the medium was removed and the cells were processed for incorporation of [^{3}H]mannose into TCA insoluble material by standard techniques and expressed as cpm incorporated per mg cell protein.

RESULTS

In the control embryos cultured for 40 h, embryonic heart beat and circulation were maintained, whereas circulation in the major blood vessels of the yolk sac ceased at 24 h of culture. The total protein content of control embryos doubled after 40 h of culture at which time the somite number corresponded to 16 h of growth *in vivo*. (Table 1). Embryos explanted on day 11 were at the stage of nasal placode invagination (Fig. 2*a*) and during the 40 h of culture, formation

Table 1. *Mean somite number and protein content of rat embryos explanted on late day* 11 *of gestation and cultured for* 40 *h, and rat embryos at corresponding developmental age* in vivo

Age (h after 11·0 days)	Number of embryos	Mean somite number ± S.E.	Mean protein content ± S.E. (μg)
0 (at explantation)	16	35·4 ± 0·2	475·5 ± 19·2
16 (*in vivo*)	17	44·1 ± 0·2	1535·0 ± 50·1*
40 (*in vitro*)	16	44·9 ± 0·8	1144·0 ± 70·9*

* $P < 0{\cdot}001$.

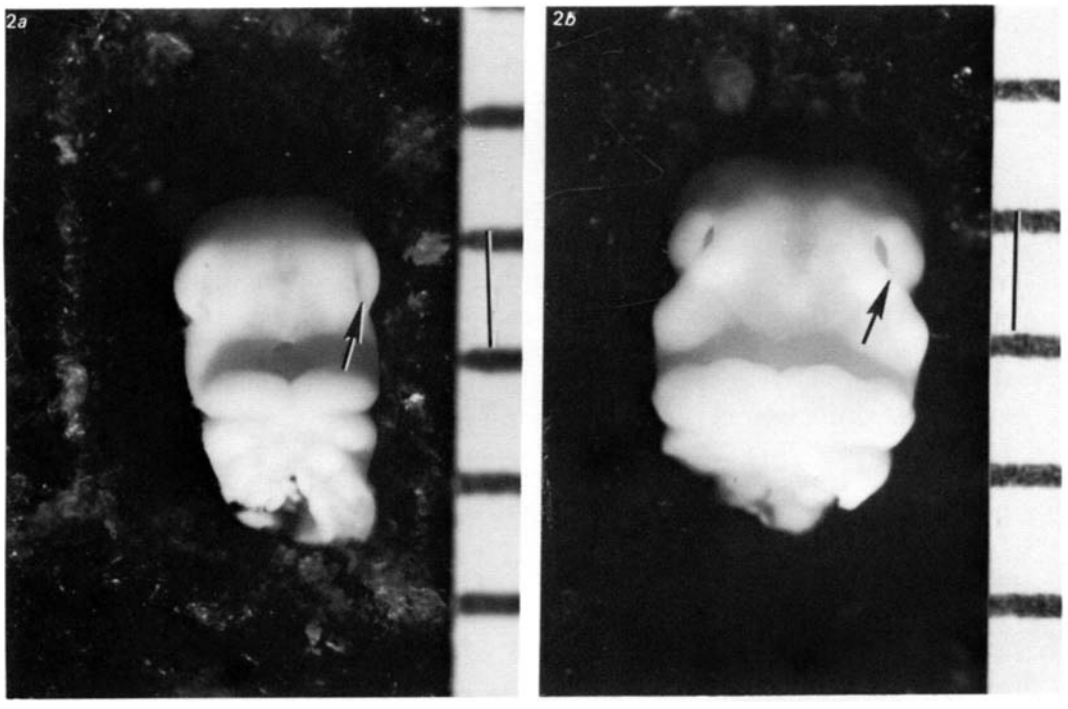

Fig. 2(*a*) An inferior view of rat embryo head explanted on late day 11. The arrow indicates the slightly invaginated nasal placode. Bar represents 1 mm. 2(*b*) An inferior view of rat embryo head cultured for 40 h. The arrow indicates the fused nasal processes. Bar represents 1 mm.

of the lateral and medial nasal process took place and then these opposing nasal processes made contact and fused each other (Fig. 2*b*).

In 93% of those embryos in which the nasal placode was impaled with a TM-soaked eyelash (14 out of 15 embryos), there was an obvious early stage in the formation of cleft lip (Fig. 3). The cells observed (Fig. 4*a*) near the eyelash in the treated embryos probably represent blood components released at the time of eyelash implantation. It appeared that in the majority of these affected

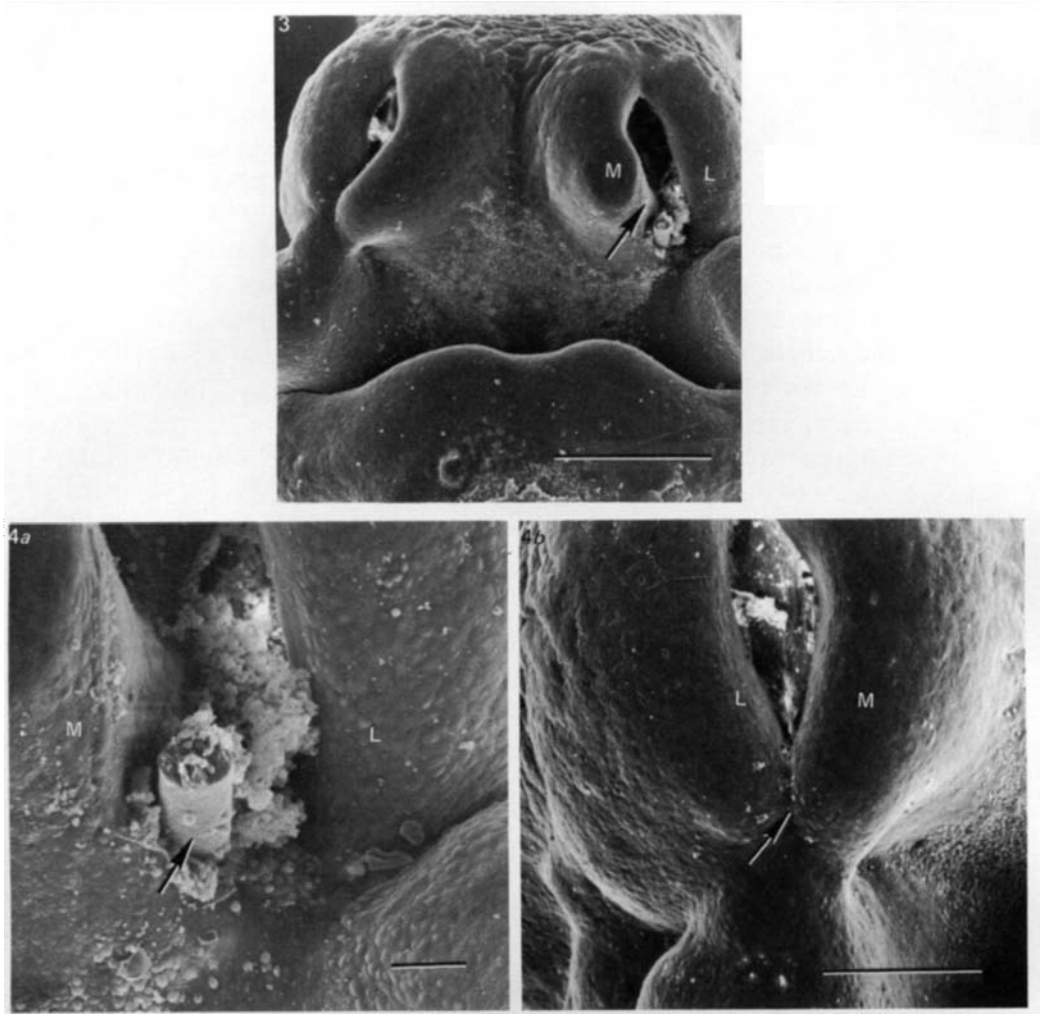

Fig. 3. An inferior view of rat embryo head cultured for 40 h. The arrow indicates the cleft between medial and lateral nasal processes in treated side with TM. M, medial nasal process; L, lateral nasal process. Bar represents 500 μm.

Fig. 4(*a*) A higher magnification of Fig. 3. The arrow indicates the implanted eyelash in the cleft in treated side with TM. M, medial nasal process; L, lateral nasal process. Bar represents 50 μm. (*b*) A higher magnification of Fig. 3. The arrow indicates the fused nasal processes in control side where eyelash was also implanted without TM. M, medial nasal process; L, lateral nasal process. Bar represents 250 μm.

embryos, the medial nasal process was somewhat smaller and less developed than the lateral nasal process. Fusion occurred normally in all of the 15 nasal placodes (contralateral to the TM-treated placodes) that were impaled with eyelashes soaked in methanol without TM (Fig. 4*b*).

A bioassay was used to estimate the amount of tunicamycin bound to the eyelash soaked in TM. This assay was based on the incorporation of labelled

mannose into glycoproteins of cultured chick embryo fibroblasts. It is known from the studies of Olden *et al.* (1978) that tunicamycin present in such cultures at levels of 0·05 μg/ml causes a dramatic (> 95 %) inhibition of labelled mannose incorporation into glycoproteins without substantially altering protein synthesis. In the present study, it was found that 93×10^3 cpm/mg protein were incorporated in the control cultures compared to $3{\cdot}7 \times 10^3$ cpm/mg protein in the presence of 0·05 μg/ml tunicamycin. In the presence of the tunicamycin released from the eyelash in culture, the incorporation was decreased to $6{\cdot}4 \times 10^3$ cpm/mg. Assuming that all the tunicamycin was released from the eyelash under these conditions, there was approximately 20 ng bound to each eyelash.

It was also observed that eyelashes soaked in 0·05 mg/ml of TM did not result in cleft lip whereas those soaked in 1 mg/ml produced 100 % cleft lip foetuses with a more severe effect on the growth of the medial nasal process. It therefore appears that TM exerts a dose-dependent effect when administered locally, whereas when it was added directly to the culture medium at 0·05–0·2 μg/ml for the entire 40 h, (results not shown) a large percentage of foetal deaths were observed.

DISCUSSION

The techniques for whole-embryo culture have advanced considerably over the past several years (New, 1978). It is now possible to culture through the major period of mammalian organogenesis, including formation of the primary palate (New, 1978). The development of the primary palate is not well understood, but appears to involve similar events as occur during secondary palate formation, such as epithelial cell contact, adhesion and programmed cell death (Greene & Pratt, 1976).

The ability to culture rodent embryos during this time allows one to ask certain questions concerning basic developmental mechanisms and the influence that various drugs or chemicals may have when added in culture. This would allow for the testing of teratogenic effects directly without maternal metabolism or placental transfer variations in the amount of drug exposed to the foetus. Unfortunately, many teratogens of interest are inhibitors of DNA, RNA and protein synthesis when added directly to the culture medium, and often cause death of the foetus (Eto & Horigan, 1977). To overcome this problem, it would be desirable to have a technique whereby small amounts of a teratogen could be administered locally to the tissue under investigation. Wilk (1969) reported that small pieces of Millipore filters soaked in chlorcyclizine and inserted between the uterus and visceral yolk sac *in vivo* resulted in cleft palate formation in the rat. Previous studies (Eto & Horigan, 1977) have shown that microinjection of colchicine was possible into the region of the developing primary palate in culture, but due to leakage of the solution and the production of widespread tissue damage, this approach does not appear to be promising.

The results of the present study, however, demonstrate a promising new

approach to this problem by using a chemically-impregnated eyelash. The results with tunicamycin (TM) were highly reproducible. Little tissue damage was observed and the effect of TM was restricted to the region of the primary palate. Tunicamycin is an inhibitor of protein glycosylation that has specific effects in both cell and organ culture (Takatsuki & Tamura, 1971; Kuo & Lampen, 1974; Schwarz, Roheschnerder & Schmidt, 1976; Olden, Pratt, Jaworski & Yamada, 1979a; Pratt, Yamada, Olden, Ohamian & Hascall, 1979; Thesleff & Pratt, 1980). The most widely studied effect of tunicamycin is on fibronectin (FN) where TM causes decreased amounts of FN on the cell surface and in the extracellular matrix (Olden, Pratt & Yamada, 1979*b*).

Recently, it has been shown that TM inhibits the fusion of embryonic chick myoblasts in cell culture (Olden, Law, Hunter & Parent, 1981) and the differentiation of rodent odontoblasts in organ culture (Thesleff & Pratt, 1980). In both systems, the effects of TM appear to be related to an alteration of cell surface glycoproteins. In the present study, TM causes a dose-dependent formation of cleft lip in whole-embryo culture. TM appears to primarily affect the growth of the medial nasal process which prevents its fusion with the lateral nasal process. This effect of TM may result from an alteration in fibronectin in the mesenchyme of the developing primary palate (Silver & Pratt, 1979) or may also result from a growth inhibitory effect of TM under these conditions. The exact amount of TM released from the eyelash into the developing facial processes is not known, but the effects of TM are limited to this region of the embryo and do not result in embryonic death. Presumably other agents, including labelled drugs, can be absorbed onto an eyelash or other suitable implantable material and quantitated by similar techniques as reported here.

The results of this study demonstrate that primary palate development can be altered with localized administration of a teratogen and represents an approach that may extend our knowledge of the events surrounding development of the primary palate. This technique should certainly be further investigated with other drugs and developing tissues.

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