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Growth and Differentiation of the Fetal Mouse Palate Cultured *In Vitro*: Comparison of *In Vitro* Development of Day-12.5 and Day-13.5 Fetal Palates

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SUMMARY

The palatal regions of days-12.5 and -13.5 mouse fetuses were cultivated in a chemically-defined medium, and their *in vitro* development was compared at intervals during incubation.

After a 24-hr culture of day-12.5 palates, palatal shelves became elevated and grew towards the midline, which was faster than *in vivo* palatogenesis between day 12.5 and day 13.5 of gestation. After a 48-hr culture of day-12.5 palates, the opposing shelves came into contact and/or fused with each other in 19% of the cases and partially fused in 6%; after a 24-hr culture of day-13.5 palates, contact and/or fusion of the shelves occurred in 88% and partial fusion in 6%. After a 72-hr culture of day-12.5 palates, palatal shelves completely fused in 89%; after a 48-hr culture of day-13.5 palates, shelves completely fused in 31%. After a 96-hr culture of day-12.5 palates, the fusion rate was similar to that of the 72-hr culture, suggesting that the rate reached the maximum after 72 hr. By a 72-hr culture of day-13.5 palates, all the shelves completely fused. Although the *in vitro* development of day-12.5 fetal mouse palates was somewhat slower than *in vivo* palatal development, the process of palate fusion *in vitro* simulated the *in vivo* palatogenetic process. In conclusion, the 72-hr culture of day-12.5 fetal mouse palates may be most suitable for studying normal and abnormal palatogenesis *in vitro* and for *in vitro* teratogen screening.

Key words: palatogenesis, mouse fetus, secondary palate, organ culture, *in vitro*

INTRODUCTION

Many studies have been conducted on *in vitro* development of fetal mouse palates[1–3] and the effects of various chemicals on cultured fetal palates[4–9]. In most of these studies, they employed a static culture technique and succeeded in inducing the fusion the palatal shelves *in vitro* by cultivating day-13 or day -14 fetal mouse palates. However, *in vitro* fusion of day-12.5 fetal mouse palates has not always between possible; a 72-hr culture failed to induce the complete fusion of palatal shelves[1], while a 96-hr culture with exchanges of the culture medium succeeded in inducing the fusion of palatal shelves[9].

Recently, Shiota et al.[10] developed a novel technique to cultivate fetal mouse palates in a chemically-defined serum-free medium by suspension culture. In their study, the secondary palate of day-12.5 mouse fetuses closed successfully after a 72-hr culture and the *in vitro* palatogenesis was essentially similar to the *in vivo* development. A further study where day-12.5 fetal palates were cultivated for 24, 48, or 72 hrs showed that although the process of the palate fusion *in vitro* was found to simulate the *in vivo* palatogenetic process, the explants cultivated *in vitro* did not grow in size and the *in vitro* development was slower than that occurring *in vivo*.[11]

In the present study, palatal primordia of day-12.5 and day-13.5 mouse fetuses were cultivated for up to 96 hr and 72 hr, respectively, and the *in vitro* palatal development was compared at intervals during incubation.

MATERIALS AND METHODS

Animals

Slc:ICR mice were purchased from Japan SLC, Inc. (Shizuoka). At the age of 8 to 13 weeks, each virgin female was mated overnight with a male from the same stock, and the day on which a vaginal plug was found was designated as day 0 of pregnancy. The midnight (0:00) of day 0 of pregnancy was considered as the start of gestation. Mice were given food pellets (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

Organ culture method

Between 10:00 and 12:00 on day 12 or 13 of gestation, pregnant females were killed by cervical dislocation and their uterus was aseptically removed. The fetuses were transferred into a Petri dish and their maxillary region was dissected with a pair of scalpels and forceps. The dissected maxillary regions including palatal primordia were then cultivated in a 50 ml penicillin bottle containing 8 ml of the culture medium which had been sterile-filtered. The composition of the culture medium was the same as that described elsewhere[10]. No serum or antibiotics were added to the medium. Three or 4 explants of day-12.5 fetuses or 2 or 3 explants of day-13.5 fetuses were put into one bottle. Each bottle was sealed airtight with a rubber stopper and a metal clamp. The bottles were flushed for approximately 2 min with a gas mixture of 50% O_2, 5% CO_2, and 45% N_2 using syringe needles. Day-12.5 fetal palates were then cultivated for 24, 48, 72, and 96 hr and day-13.5 palates for 24, 48, and 72 hr at 38°C on a roller device (20–25 rpm). During the culture period, the bottles were flushed every 24 hr with the same gas mixture. The medium was not changed during the culture period.

Observation and measurement of explants

At the end of the culture period, the palatal explants were washed gently with physiological saline solution, fixed overnight in Bouin's fluid, and stored in 70% ethanol until further examination. Each palate was measured under a dissection microscope equipped with an ocular micrometer; the length of the palatal shelf was recorded, and the length of the fused portion of palatal shelves was also measured in cases where palatal shelves were fusing. The ratio of the length of fused portion to the length of palatal shelf (LPS/LFP) was

Table 1. Types and frequencies of palatal closure of day-12.5 and day-13.5 fetal mouse

Age of fetuses (day)	Time of incubation (hr)	Number of palates examined	Length of palatal shelf (mm): mean±S.D.	Length of fused portion (mm): mean±S.D.	LFP/LPS[a] (%): mean±S.D.
12.5	24	18	0.99±0.19	0	0
	48	16	0.99±0.06	0.04±0.08	3.5± 7.7
	72	19	1.24±0.21	0.88±0.23	75.3±13.8
	96	18	1.15±0.12	0.72±0.39	60.7±31.4
13.5	24	16	1.41±0.09	0.02±0.09	1.5± 5.9
	48	13	1.20±0.11	0.34±0.48	27.2±38.1
	72	9	1.47±0.07	1.45±0.09	98.4± 3.6

a) LFP/LPS: Length of fused portion of palatal shelves/Length of palatal shelf.
b) No contact of the shelves.
c) Contact but no fusion of the shelves.
d) Partial fusion (<1/2 of palatal length) of the shelves.
e) Complete fusion (>1/2 of palatal length) of the shelves.

calculated. The stages of palate fusion were classified as "fused", "in contact but not fused", or "not in contact". Palates were so classified as "in contact but not fused" if the shelves in contact were readily separated at the midline with forceps. The fused palates were further classified into "completely fused" or "partially fused"; "completely fused" was applied when more than 1/2 of the total length of the palatal shelf was fused, and "partially fused" was applied when less than 1/2 of the total length of the palatal shelf was fused.

RESULTS AND DISCUSSION

Cultured palates were observed and evaluated after culture for 24, 48, 72, and 96 hr for day-12.5 fetal palates and after culture for 24, 48, and 72 hr for day-13.5 palates. Table 1 summarizes the results.

The stages of the secondary palate development of day-12.5 or day-13.5 fetal mouse palates were assessed according to the scoring system proposed by Walker and Frasser[12] and Biddle[13]. On day-12.5, fetal mouse palates were at stage 1 or earlier stages of palate development, in which palatal shelves were vertical with straight medial edges and the shelf elevation had not begun. Day-13.5 fetal palates were either at stage 2 when the posterior ends of the palatal shelves were horizontal or at stage 3 when one of the shelves was completely horizontal. After a 24-hr culture of day-12.5 fetal palates, opposing palatal shelves were in contact with each other in 1 of the 18 explants (6%), but not in the remaining 17 explants. Although those *in vitro* fetal palates corresponded to the *in vivo* day-13.5 fetal palates in the normal course of development, the contact of palatal shelves did not occur in any of the *in vivo* day-13.5 fetal palates[11].

palates cultured *in vitro* for various incubation periods

Type of palatal closure (%)			
No contact[b]	Contact[c] and/or fusion	Fusion	
		Partial[d]	Complete[e]
94.4	5.6	0	0
81.2	18.8	6.3	0
0	100.0	5.3	89.4
0	100.0	11.1	72.2
12.5	87.5	6.3	0
15.4	84.6	7.7	30.8
0	100.0	0	100.0

After a 48-hr culture of day-12.5 fetal palates (16 explants), contact and/or fusion occurred in 3 explants (19%) (one of them was partially fusing), but palatal shelves were not in contact with each other in the remaining 13 explants (81%). After a 24-hr culture of day-13.5 fetal palates which corresponded to the 48-hr culture of day-12.5 fetal palates, contact and/or fusion occurred in 14 explants (88%) (one of them was partially fusing), and no contact occurred in 2 explants (13%), indicating that they grew faster than the day-12.5 fetal palates cultured for 48 hr.

After a 72-hr culture of day-12.5 fetal palates, all the 19 explants had either come into contact or were fused (contact in 1 explant (5%), partial fusion in 1 (5%), and complete fusion in 17 (89%)). By the corresponding 48-hr culture of day-13.5 fetal palates (13 explants) contact or fusion occurred in 11 explants (85%), partial fusion in 1 (8%), complete fusion in 4 (31%), and no contact in 2 (15%). The fetal palates of ICR strain mice were completely fused *in vivo* on day 15.5 of gestation, which corresponds to day-12.5 for fetal organs cultured for a 72-hr and day-13.5 for fetal organs cultured for 48 hr. The fusion rate was 94.7% after a 72-hr culture of day-12.5 fetal palates and as low as 38.5% after a 48-hr culture of day-13.5 fetal palates, suggesting that *in vitro* development of fetal mouse palates is significantly retarded as compared with *in vivo* palatogenesis.

At the time of explantation of palatal regions in the present study, fetuses were selected by discarding those with significantly

advanced or delayed growth. However, the stage of palate closure was variable among the explants at the time of observation, which might be due to a variation in growth rate of fetal palates among individuals even within a given litter.

After a 96-hr culture of day-12.5 fetal palates (18 explants), the fusion occurred in 15 explants (83%) and in the remaining 3 (17%), contact of shelves but no fusion had occurred. This suggests that the fusion rate of cultivated palates of day-12.5 fetuses reaches a maximum after a 72-hr culture. On the other hand, all of the 9 explants of day-13.5 fetal palates had become completely fused after a 72-hr culture, and the LFP/LPS ratio was 98%, indicating that the complete fusion was delayed by ca. 24 hr as compared with the *in vivo* day-15.5 fetal palates and *in vitro* development of day-12.5 fetal palates. The length of the palatal shelf, which is an index of palatal growth, was not significantly different between day-12.5 and day-13.5 palates even when the incubation period was extended. However, the length of the fused portion of palatal shelves and the LFP/LPS ratio increased as the incubation period was extended.

Vargas[1] removed fetal palates from mice of days 12.5, 13.5, and 14.5 of gestation, and cultivated the palates for 24, 48, or 72 hr in a semi-defined medium with a static culture method (medium replaced daily). In day-12.5 fetal palates, 25% of the explants showed the partial fusion within 72 hr and complete fusion was not observed. In day-13.5 fetal palates, a 24-hr culture resulted in partial fusion in 90% and in complete fusion in 10%; a 48-hr culture resulted in partial fusion in 25% and in complete fusion in 75%; a 72-hr culture resulted in complete fusion in all explants. In day-14.5 fetal palates, all explants showed complete fusion within 72 hr.

Abbott et al.[9] cultivated day-12.5 fetal mouse palates for 96 hr by replacing the medium every 24 hr and obtained complete fusion in 93% and partial fusion in 7% of the control.

Thus, the suspension organ culture method using a roller device[10,11] is considered to be superior to static organ culture techniques.

We compared the *in vitro* development of day-12.5 and day-13.5 fetal mouse palates, and higher fusion rates were obtained in day-13.5 fetal palates than in day-12.5 fetal palates which were cultured for 24 hr longer. Rapid growth of palatal shelves occurred between the 48 and 72 hr after culture both in days-12.5 and -13.5 fetal palates. This suggests that a certain length of time is required for palatal shelves to become adapted to the *in vitro* environment and begin to develop.

Normal development of the secondary palate follows the 4 distinct stages, (1) growth of the palatal shelves, (2) reorientation of the shelves, (3) contact of the medial edge, and (4) fusion of the shelves[14]. Many drugs are thought to induce cleft palate by interfering with any one of these 4 stages[15]. Our organ culture method to cultivate day-12.5 fetal mouse palates for 72 hr might be useful to study the mechanisms of chemically-induced cleft palate formation and screen the development toxicity of new chemicals.

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References

1) Vargas, V.I. (1967) Palatal fusion *in vitro* in the mouse. *Archs Oral Biol.*, **12**: 1283–1288.
2) Chaudhry, A.P. and Siar, S. (1967) *In vitro* study of fusion of palatal shelves in A/Jax mouse embryos. *J. Dent. Res.*, **46**: 257–260.
3) Smiley, G.R. and Koch, W.E. (1971) Fine structure of mouse secondary palate development *in vitro*. *J. Dent. Res.*, **50**: 1671–1677.
4) Lahti, A., Antila, E. and Saxen, L. (1972) The effect of hydrocortisone on the closure of the palatal shelves in two inbred strain of mice *in vivo* and *in vitro*. *Teratology*, **6**: 37–42.

5) Saxén, L. (1973) Effects of hydrocortisone on the development *in vitro* of the secondary palate in two inbred strains of mice. *Archs. Oral Biol.*, **18**: 1469–1479.
6) Baird, G. and Verrusio, A.C. (1973) Inhibition of palatal fusion *in vitro* by β-2-thienyalanine. *Teratology*, **7**: 37–48.
7) Fairbanks, M.B. and Kollar, E.J. (1974) Inhibition of palatal fusion *in vitro* by hadacidin. *Teratology*, **9**: 169–178.
8) Newall, D.R. and Edwards, J.R.G. (1981) The effect of vitamin A on fusion of mouse palates. II. Retinyl palmitate, retinol, and retinoic acid *in vitro*. *Teratology*, **23**: 125–130.
9) Abbott, B.D., Logsdon, T.R. and Wilke, T.S. (1994) Effects of methanol on embryonic mouse palate in serum-free organ culture. *Teratology*, **49**: 122–134.
10) Shiota, K., Kosazuma, T., Klug, S. and Neubert, D. (1990) Development of the fetal mouse palate in suspension organ culture. *Acta Anat.*, **137**: 59–64.
11) Kosazuma, T. (1994) Growth and differentiation of the fetal mouse palate cultured *in vitro*: Comparison with *in vivo* palatogenesis. *Alter. Animal Test. Exp.*, **2**: 177–181.
12) Walker, B.E. and Fraser, F.C. (1956) Closure of the secondary palate in three strains of mice. *J. Embryol. Exp. Morph.*, **4**: 176–186.
13) Biddle, F.G. (1980) Palate development in the mouse: A quantitative method that permits the estimation of time and rate of palate closure. *Teratology*, **22**: 239–246.
14) Greene, R.M. and Pratt, R.M. (1976) Developmental aspects of secondary palate formation. *J. Embryol. Exp. Morph.*, **36**: 225–245.
15) Pratt, R.M. (1983) Mechanisms of chemically-induced cleft palate. *Trends Pharmacol. Sci.*, **4**: 160–162.