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Growth and Differentiation of the Fetal Mouse Palate Cultured *In Vitro*: Comparison with *In Vivo* Palatogenesis

Tsuneo Kosazuma

Institute of Applied Medicine, Hokkaido Radio-isotope Center, Ltd., Ishikari, Hokkaido 061-32, and Department of Hygienic Chemistry, Hoshi University, Ebara, Shinagawa, Tokyo 142, Japan

Summary

Palatal primordia of day-12.5 mouse fetuses were cultivated in a chemically-defined medium by a suspension culture technique, and their growth and differentiation *in vitro* were compared with those of the fetal palates *in vivo* at the corresponding gestational stages. The maxillary explants cultivated *in vitro* for up to 72 hr did not grow in size and remained almost in the same size as those in day-12.5 fetuses. However, the palatal shelves elevated and grew faster towards the midline until 24 hr in culture as compared with *in vivo* development. In day-14.5 fetal palates *in vivo*, the opposing palatal shelves have contacted with each other in about 80% of cases and 73% of the palates have begun to fuse, while the corresponding rates *in vitro* were 19% and 6%, respectively, suggesting that *in vitro* palatogenesis is delayed by 48 hr in culture. At 72 hr of culture, the rate of completely closed palates was 89% against the 100% closure rate for day-15.5 fetuses *in vivo*. Although the comparison showed that the *in vitro* development of the fetal mouse palate is somewhat slower than that *in vivo*, the process of palate fusion *in vitro* was found to simulate the *in vivo* palatogenetic process, and suspension organ culture appears superior to the conventional static organ culture. Suspention organ culture of fetal mouse palates should be useful in the study of normal and abnormal palatogenetic processes.

Key Words: palatogenesis, mouse fetus, secondary palate, *in vitro*, *in vivo*

Introduction

Organ culture techniques have been used to study the mechanisms of secondary palate formation, and the fetal palates of mice[1-10] and rats[11-17] have been most frequently used for *in vitro* studies. When fetal rodent palates were cultivated, palates of day-13 mouse fetuses or days-14 and -15 rat fetuses are usually used, which are not young enough to observe early stage of secondary palate formation. Furthermore, most such studies employed static culture techniques, in which palatal explants were placed on filter paper or membrane filters in a culture dish and cultivated in a CO_2 incubator. In such conventional static culture, the growth of explanted palates was often significantly delayed as compared with the normal palate growth *in vivo* and the process of palatal closure did not necessarily simulate that occurring *in vivo*.

Recently, we developed a novel technique to cultivate fetal mouse palates in a chemically-defined serumless medium by suspension culture in rotating bottles[18]. In this system, explanted fetal palates successfully closed within 72 hr, and the *in vitro* fusion of the palatal shelves simulated the palatogenetic process *in vivo* both macroscopically and histologically. However, the size of the explants was significantly smaller at the end of the 72 hr culture period as compared with the size of the fetal palate *in vivo* at the corresponding developmental stage[18].

In the present study, day-12.5 fetal mouse palates were cultivated for up to 72 hr using

the same method[18], and the growth and differentiation of the explanted palates were compared with those of the fetal palates *in vivo* at the corresponding gestational stages to clarify the merits and limitations of palate organ culture.

Materials and Methods

Animals

Slc:ICR mice, 6 weeks old, were purchased from SLC (Shizuoka, Japan) and kept in an animal room. The room temperature was maintained at 22±2°C and the relative humidity at 55±5%. The lighting was on a 12:12 hr light: dark cycle. At the age of 8 to 13 weeks, each virgin female was mated overnight with a male, and the day on which a vaginal plug was found was designated as day 0 of pregnancy. Commercially available laboratory chow (Oriental Yeast Co., Tokyo, Japan) and tap water were given ad libitum.

In vitro organ culture

Between 10 and 12 hr on day 12 of gestatioon, pregnant female mice were killed by cervical dislocation and their uteri were aseptically removed. The fetuses were transferred into a Petri dish and their maxillary region was dissected with a pair of scalpels. They were then cultivated in a chemically-defined medium for 24, 48 or 72 hr according to the method described previously[18]. No serum or antibiotics were added to the medium. The palatal explants were cultured in 50 ml penicillin bottles with 8 ml of culture medium which had been sterile-filtered. Three or four explants were put into one bottle and 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, and then incubated at 38°C on a roller device (20-25 rpm) for 24, 48 or 72 hr. During the culture period, the bottles were flushed every 24 hr with the same gas mixture. The medium was not changed for up to 72 hr. After 24, 48, and 72 hr in culture, the explants were harvested and washed in physiological saline solution, fixed overnight in Bouin's fluid, and stored in 70% ethanol until further examination.

In vivo procedures

On days 12, 13, 14, and 15 of gestation, pregnant female mice were killed by cervical dislocation between 10:00 and 12:00. The fetuses were removed from the uteri and placed in Tyrode's solution in a Petri dish. The palates were dissected under a dissection microscope as described above. They were fixed overnight in Bouin's fluid and then stored in 70% ethanol.

Observation and measurement of the palates

Each palate obtained as above was measured under a dissection microscope equipped with an ocular micrometer and the head width, the head length, and the palatal shelf length were recorded. The length of the fused portion of palatal shelves was also measured in cases where palatal shelves were fusing. The narrowest gap between the opposing palatal phelves was also measured when palatal shelves have not contacted with each other. The stages of palate fusion were classified as "fused", "contacted but not fused", or "not contacted." Palates were classified as "contacted but not fused" if the contacted shelves were readily separated at the midline with forceps. The fused palates were further classified into "completely fused" or "partially fused." The term "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.

The frequency or average value of each parameter was obtained for each experimental group and the data were compared with those of the corresponding *in vivo* controls. The mean values were compared using Student's t-test, and the frequencies were compared by using chi-square test with

Yates' correction.

Results and Discussion

The palatal explants were measured and the palatogenetic process, i.e. the contact and fusion of the opposing palatal shelves, was evaluated after 24, 48, and 72 hr culture. Data are shown in Tables 1 and 2 in comparison with *in vivo* palatogenesis at the corresponding gestational stages.

In fetal mice *in vivo*, the palatal shelves grow to bring about palate fusion, and their head width, head length and palatal shelf length increase as gestational age advances. However, the size of the palate cultivated *in vitro* remained almost unchanged even after 72 hr in culture. Therefore, the palates cultivated for 24, 48 or 72 hr *in vitro* were significantly smaller than their respective *in vivo* controls.

The gap between the opposing palatal shelves was significantly smaller in the palates cultured *in vitro* for 24 hr than that in day-13.5 fetal palates grown *in vivo*. Within 24 hr in culture, contact of the palatal shelves occurred in 1 of 18 explatns (5.6%), while palatal shelf contact had occurred in none of day-13.5 palates *in vivo*. Of 16 explants cultured for 48 hr, partial fusion was observed in 1 (6.3%) and contact in 2 cases (12.5%), but palatal shelves had not contacted with each other in the remaining 13 cases (81.2%). On the other hand, among day-14.5 fetuses grown *in vivo* (N=48), complete palate fusion was observed in 27 (56.3%), partial fusion in 8 (16.7%), contact without fusion in 2 (4.2%), and no contact in 11 (22.9%). The length of the fused portion and the fused portion/palatal length (LFP/LPS) ratio at this stage significantly smaller *in vitro* than the corresponding figures *in vivo*. By 72 hr in culture, palatal shelves fused with each other in about 95% of the cases and most of these were completely fused. All the fetal palates had fused *in vivo* on day 15.5 of gestation. Thus, the *in vitro* development of palatal shelves appears to be faster during the first 24 hr in culture than *in vivo*, but *in vivo* palatal development exceeds *in vitro* development during the later half of the culture period. The fusion rate after 72 hr culture was 95%, which was lower than but

Table 1. Comparison of *in vivo* and *in vitro* development of fetal mouse palates

Age of fetuses (day)		12.5	13.5	14.5	15.5
Time of incubation (hr)		0	24	48	72
Number of palates examined	*in vivo*	46	41	48	24
	in vitro		18	16	19
Head width (mm)	*in vivo*	2.91±0.16[a]	3.60±0.11	4.55±0.13	5.02±0.16
	in vitro		2.58±0.26[d]	2.57±0.07[d]	2.64±0.17[d]
Head length (mm)	*in vivo*	3.08±0.29	3.93±0.13	5.03±0.26	5.76±0.69
	in vitro		2.82±0.40[d]	2.66±0.13[d]	2.77±0.36[d]
Narrowest gap (mm)	*in vivo*	0.46±0.06	0.40±0.05	0.24±0.22	0
	in vitro		0.16±0.13[e]	0.09±0.05	0
Gap/head width (%)	*in vivo*	15.9±1.8	11.1±1.7	5.7±4.4	0
	in vitro		6.7±6.2	2.7±1.9	0
Length of palatal shelf (mm)	*in vivo*	1.15±0.07	1.57±0.11	2.17±0.14	2.62±0.09
	in vitro		0.99±0.19[d]	0.99±0.06[d]	1.24±0.21[d]
Length of fused portion (mm)	*in vivo*	0	0	1.35±0.49	2.62±0.09
	in vitro		0	0.04±0.08[e]	0.88±0.23[d]
LFP[b]/LPS[c] (%)	*in vivo*	0	0	60.0±18.3	100.0±0.0
	in vitro		0	3.5±7.7[d]	75.3±13.8[d]

a) Mean±SD.
b) LFP: Length of the fused portion of palatal shelves.
c) LPS: Length of the palatal shelf.
d) Significantly different from the *in vivo* group ($p<0.01$).
e) Significantly different from the *in vivo* group ($p<0.05$).

Table 2. **Types and frequency of *in vivo* and *in vitro* closure of the fetal mouse palates**

Age of fetuses (day)		12.5	13.5	14.5	15.5
Time of incubation (hr)		0	24	48	72
Number of palates examined	*in vivo*	46	41	48	24
	in vitro		18	16	19
Palate closure (%)					
No contact[a]	*In vivo*	100	100	22.9	0
	In vitro		94.4	81.2[e]	0
Contact[b] or fusion	*In vivo*	0	0	77.1	100
	In vitro		5.6	18.8[e]	100
Fusion					
Partial[c]	*In vivo*	0	0	16.7	0
	In vitro		0	6.3	5.3
Complete[d]	*In vivo*	0	0	56.3	100
	In vitro		0	0	89.4

a) No contact of the shelves.
b) Contact but no fusion of the shelves.
c) Partial fusion (< 1/2 of the palatal length) of the shelves.
d) Complete fusion (≧ 1/2 of the palatal length) of the shelves.
e) Significantly different from the *in vivo* group ($p<0.01$).

not significantly different from the corresponding rate *in vivo*. The opposing shelves in cultured palates elevated and grew towards the midline faster than *in vivo* during the first 24 hr probably because the removal of the tongue may have facilitated shelf elevation.

Palate fusion occurred in about 95% of cases in our suspension culture. *In vitro* culture of fetal rodent palates has been attempted previously by many investigators, but the rates of palate fusion in their system were poorer compared with the present results. For example, Varges[1] dissected the palates from fetal mice on days 12.5, 13.5 and 14.5 of gestation and cultivated them for 24, 48, or 72 hr in a semi-defined medium by a static culture method. In this previous study, no complete fusion was observed in day-12.5 fetal palates cultured for 72 hr, while complete fusion occurred in those of day-13.5 and -14.5 fetal palates cultured for 24-72 hr. Thus, the present suspension culture method seems to be superior to conventional static organ culture, although the palate fusion rate is not as good as the corresponding rate *in vivo*.

Although the palatogenetic process in the present suspension organ culture system simulated the *in vivo* process, some necrosis was observed in the nasal septum and the deep portion of the maxillary processes[18]. This was probably due to the insufficient supply of nutrients and/or oxygen to the deeper tissue in explants, since these are provided only by means of diffusion in organ culture.

In summary, the present study showed that suspension culture of fetal mouse palates is superior to static organ culture techniques and has some advantages for the study of normal and abnormal palatogenesis, although some limitations of such *in vitro* technique should be taken into account.

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