ABSTRACT Palatal primordia of day-12.5 ICR mouse fetuses were cultured in a chemically-defined serumless medium by a suspension culture technique, and the developmental toxicity of aspirin and its metabolites on in vitro palatogenesis was studied. Explanted fetal palates were exposed in vitro for 72 hr to 0.5–2 mM aspirin (ASP), 0.25–2 mM salicylic acid (SA), 0.5–2 mM salicyluric acid (SUA), 1–2 mM 2,3-dihydroxybenzoic acid (3DHB), or 1–2 mM 2,5-dihydroxybenzoic acid (5DHB). After 72 hr culture, ASP at 2 mM and SA at 0.25 mM inhibited the growth and fusion of palatal shelves, and SUA at 1 mM prevented palatal fusion. On the other hand, 3DHB and 5DHB did not exert any significant toxic effects on cultured palates at concentrations up to 2 mM. Judging from the 50% inhibitory concentration (IC50), SA (IC50 = 0.9 mM) was the most toxic of the 5 compounds tested, with a decreasing order of ASP (IC50 = 1.5 mM), SUA (IC50 = 1.6 mM), and DHBs (IC50 = over 2 mM for both 3DHB and 5DHB). With respect to developmental toxicity, cultured fetal mouse palates showed the susceptibility to aspirin and its metabolites which is intermediate between the susceptibility of rat embryos in vivo and that of postimplantation rat embryos cultured in vitro. The significance of fetal organ culture for evaluating developmental toxicity of chemicals is also discussed.

Key words: aspirin, salicylates, fetal mouse palate, organ culture, in vitro
Aspirin (ASP) has been clinically used as an analgesic, antipyretic, anti-inflammatory, and anti-rheumatic agent. In humans, ASP is easily hydrolyzed to salicylic acid (SA) and further converted to salicylic acid (SUA), salicyloyl acyl gluturonide, salicylic phenol glucuronide, 2,3-dihydroxybenzoic acid (3DHB), 2,5-dihydroxybenzoic acid (5DHB), and 2,3,5-tri-hydroxybenzoic acid (for review, see Gabrielsson and Larsson, 1987). This metabolic pathway is considered the same both in the human and rat (Levy and Leondards, 1966).

ASP has been shown to be teratogenic in laboratory animals and induce various malformations such as cleft lip, exencephaly, spina bifida, and microcephaly (Shepard, 1989). Kimmel et al. (1971) suggested that the causative agent for ASP teratogenesis was SA, since only SA was found in the embryonic tissue and maternal serum following oral administration of ASP to pregnant rats. SA (Tanaka et al., 1973) and its sodium salt sodium salicylate (Warkany and Takacs, 1959; Larsson et al., 1963; Minor and Becker, 1971) have been shown to be teratogenic in rodents, but other metabolites such as SUA, 3DHB, 5DHB, and 2,3,5-trihydroxybenzoic acid were not found teratogenic when they were administered to pregnant rats (Koshakji and Schulert, 1973).

Saito et al. (1982) showed that the teratogenicity of ASP in rats decreased with the elevation of maternal metabolic activity of the drug, indicating that ASP itself or some of its intermediate metabolites may be responsible for the teratogenesis. Salicylates have also been shown to inhibit the synthesis of acid mucopolysaccharides in mouse embryos (Larsson and Boström, 1965) and the activities of DNA and RNA polymerases in rat fetuses (Janakidevi and Smith, 1969), which may be related to their teratogenic activity. However, the mechanisms how salicylates damage embryonic tissues and cause developmental abnormalities are virtually unknown.

In vitro toxicity of salicylates on cultured cells and explanted embryos has been studied using various in vitro experimental systems. ASP at high concentrations inhibited the in vitro differentiation and survival of limb bud cells and midbrain cells of rat fetuses (Flint and Orton, 1984; Uphill et al., 1990). In another study using human embryonic palatal mesenchyme cells, ASP was found not to inhibit the growth of the cells in vitro, although it causes cleft palate in vivo (Pratt and Willis, 1985). Sodium salicylate inhibited the aggregation, growth and differentiation of chick embryo neural retina cells cultured in vitro (Daston et al., 1991). It has also been shown that ASP, SA, sodium salicylate, 3DHB, 5DHB, and SUA adversely affect the in vitro development of explanted rodent embryos and induce various abnormalities in cultured embryos (McGarry et al., 1981; Greenaway et al., 1982, 1984; Yokoyama et al., 1984; Cicurel and Schmid, 1986).

Recently, we developed a suspension culture technique of fetal mouse palates during a critical period of palatogenesis in a chemically-defined serumless medium (Shiotani et al., 1990). In this system, explanted fetal palates successfully closed within 72 hr and the in vitro fusion of the palatal shelves simulated the palatogenic process occurring in vivo. In the present study, we investigated the effects of ASP and its major metabolites on the in vitro development of fetal mouse palates and compared their toxic potentials.

**MATERIALS AND METHODS**

Slc:ICR mice of 6 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and kept in an animal facility. 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. Commercially available laboratory chow (MF, Oriental Yeast Co., Tokyo) and tap water were given ad libitum. At the age of 8–13 weeks, each virgin female was mated.
In vitro toxicity of salicylates

Aspirin (ASP), salicylic acid (SA), and 2,5-dihydroxybenzoic acid (5DHB) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka) and salicyluric acid (SUA) and 2,3-dihydroxybenzoic acid (3DHB) were from Sigma Chemical Co. (U.S.A.). Each chemical was dissolved in ethanol to make stock solutions. The concentration of each stock solution was adjusted so that the final volume added to the culture medium was

overnight with a male mouse and the day on which a vaginal plug was found was designated as day 0 of pregnancy.

Between 10:00 and 12:00 on day 12 of gestation, pregnant females were killed by cervical dislocation and the fetuses were aseptically removed from the uterus. The fetuses were transferred into a Petri dish and their maxillary region including the palatal primordium was dissected with a pair of scalpels. They were then cultivated in a 50 ml penicillin bottle which contained 8 ml of sterile-filtered culture medium and a fixed volume of stock solutions of each drug. The composition of the culture medium was the same as described previously (Shiota et al., 1990; Kosazuma et al., 1994). No serum or antibiotics were added to the medium. Three or 4 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% O₂, 5% CO₂, and 45% N₂. The bottles were then incubated at 38°C on a roller device (20–25 rpm) for 72 hr. During the culture period, the bottles were flushed every 24 hr with the same gas mixture. The medium was not changed during the cultivation period.

Fig. 1 Measurements in cultured palates. PL palatal shelf length, FL length of the fused portion, NG narrowest gap (when palatal shelves are not fused).
5–20 µl per bottle. Twenty microliter (20 µl) of the vehicle (ethanol) was added to each control bottle. At the end of the culture period, the explants were washed in physiological saline solution, fixed overnight in Bouin fluid, and stored in 70% ethanol until further examination.

The palatal explants were observed and measured under a dissection microscope equipped with an ocular micrometer, and the length of palatal shelves was recorded. The length of the fused portion of palatal shelves was also measured when opposing palatal shelves were fusing (Fig. 1). The stages of palatal 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. In fusing palates, the ratio of the length of the fused portion to the total shelf length (LFP/PSL) was also calculated.

The frequency or average value of each parameter was obtained for each experimental group and the data were compared with the control group. Mean values were compared by using Student’s t-test, and frequencies were compared by using chi-square test with Yates’ correction. The IC_{50} values (concentrations inhibiting in 50% of the cases) was calculated by the Probit method.

RESULTS

The data on the growth and differentiation of day-12.5 fetal mouse palates cultured with ASP and its 4 major metabolites are summarized in Table 1.

In the control group where the culture medium was supplemented with the vehicle ethanol, the opposing palatal shelves came in contact in all the explants and palatal fusion was achieved in 84% of the cases. ASP did not significantly inhibit the in vitro development of fetal mouse palates at concentrations of 0.5 and 1 mM. At 2 mM, however, the palatal shelf length and the length of the fused portion were significantly shorter as compared with the control. The frequency of contacted and/or fused palates and that of fused palates in the 2 mM ASP group also decreased significantly as compared with the corresponding control values. The opposing palatal shelves failed to come in contact in 37% of the cases at 2 mM, which was significantly higher than in controls. All the parameters examined showed a concentration-dependent relationship at 0.5–2 mM.

SA at a concentration of 0.125 mM had no observable toxic effects on the in vitro development of fetal palates. As compared with the control, the growth of the palatal shelf as indicated by its length was suppressed at concentrations of 0.5–2 mM and palatal fusion was also inhibited at 0.25–2 mM. The LFP/PSL ratio also decreased significantly at 0.25–2 mM. Although palatal fusion was not significantly affected at 0.5–2 mM, that contact of opposing palatal shelves was significantly inhibited. The dose-response relationship was not obvious at concentrations over 0.5 mM, suggesting that the toxic effect reached a “plateau” at these concentrations.

SUA at a concentration of 0.5 mM exerted no inhibitory effects on the growth and fusion of palatal shelves. Although the growth of palatal shelves was not significantly affected and the opposing palatal shelves came in contact in all the explants at concentrations up to 2 mM, palatal fusion was inhibited at 1 and 2 mM in a concentration dependent manner. Both 3DHB and 5DHB exerted no significantly toxic effects on the in vitro growth and fusion of cultured palates at concentrations up to 2 mM.

The IC_{50} values for the compounds tested as calculated by the Probit method were 1.5 mM (278 µg/ml) for ASP, 0.9 mM (122 µg/ml) for SA, 1.6 mM (321 µg/ml) for SUA, and over 2 mM (308 µg/ml) for both
Table 1  Effects of aspirin and its major metabolites on fetal mouse palates cultured in vitro

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration (mM)</th>
<th>Number of explants</th>
<th>Palatal shelf length (mm); mean ± SD</th>
<th>Length of fused portion (mm); mean ± SD</th>
<th>LFP/PSL&lt;sup&gt;a&lt;/sup&gt; (%) ; mean ± SD</th>
<th>Frequency of contacted and/or fused palates (%)</th>
<th>Frequency of fused palates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>19</td>
<td>1.09 ± 0.12</td>
<td>0.51 ± 0.30</td>
<td>46.7 ± 26.9</td>
<td>100</td>
<td>84.2</td>
</tr>
<tr>
<td>Aspirin (ASP)</td>
<td>0.5</td>
<td>15</td>
<td>1.04 ± 0.11</td>
<td>0.51 ± 0.33</td>
<td>48.6 ± 30.0</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>17</td>
<td>1.04 ± 0.10</td>
<td>0.42 ± 0.28</td>
<td>40.3 ± 27.4</td>
<td>94.1</td>
<td>76.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19</td>
<td>1.01 ± 0.09*</td>
<td>0.13 ± 0.25**</td>
<td>12.6 ± 25.2**</td>
<td>63.2**</td>
<td>21.1**</td>
</tr>
<tr>
<td>Salicylic acid (SA)</td>
<td>0.125</td>
<td>15</td>
<td>1.09 ± 0.06</td>
<td>0.53 ± 0.33</td>
<td>48.1 ± 29.4</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>18</td>
<td>1.10 ± 0.10</td>
<td>0.25 ± 0.25**</td>
<td>22.8 ± 23.4*</td>
<td>100</td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>18</td>
<td>0.94 ± 0.13**</td>
<td>0.16 ± 0.23**</td>
<td>16.4 ± 22.7**</td>
<td>94.4</td>
<td>38.9**</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>18</td>
<td>0.98 ± 0.09**</td>
<td>0.20 ± 0.30**</td>
<td>20.1 ± 28.4**</td>
<td>88.9</td>
<td>38.9**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
<td>0.97 ± 0.10**</td>
<td>0.16 ± 0.21**</td>
<td>16.6 ± 22.6**</td>
<td>88.9</td>
<td>38.9**</td>
</tr>
<tr>
<td>Salicyluric acid (SUA)</td>
<td>0.5</td>
<td>18</td>
<td>1.05 ± 0.09</td>
<td>0.55 ± 0.35</td>
<td>51.3 ± 32.0</td>
<td>100</td>
<td>77.8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>17</td>
<td>1.06 ± 0.08</td>
<td>0.44 ± 0.40</td>
<td>40.3 ± 36.6</td>
<td>100</td>
<td>58.8**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17</td>
<td>1.04 ± 0.10</td>
<td>0.19 ± 0.29**</td>
<td>18.5 ± 27.7**</td>
<td>100</td>
<td>35.5**</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic</td>
<td>1</td>
<td>12</td>
<td>1.09 ± 0.15</td>
<td>0.55 ± 0.31</td>
<td>50.2 ± 26.7</td>
<td>100</td>
<td>83.3</td>
</tr>
<tr>
<td>acid (3DHB)</td>
<td>2</td>
<td>15</td>
<td>1.10 ± 0.15</td>
<td>0.51 ± 0.26</td>
<td>45.5 ± 23.0</td>
<td>100</td>
<td>86.7</td>
</tr>
<tr>
<td>2,5-dihydroxybenzoic</td>
<td>1</td>
<td>16</td>
<td>1.06 ± 0.11</td>
<td>0.43 ± 0.32</td>
<td>40.5 ± 28.6</td>
<td>100</td>
<td>81.3</td>
</tr>
<tr>
<td>acid (5DHB)</td>
<td>2</td>
<td>15</td>
<td>1.07 ± 0.10</td>
<td>0.44 ± 0.29</td>
<td>42.3 ± 26.6</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup> LFP/PSL: Ratio of the length of fused portion to the palatal shelf length.

*, ** Significantly different from controls (* P < 0.05; ** P < 0.01).
DISCUSSION

In the present study in which fetal mouse palates were cultured and treated in vitro with ASP and its major metabolites, ASP and SA inhibited both palatal closure and fusion at 2 mM or lower concentrations, while SUA, 3DHB and 5DHB specifically inhibited palatal fusion without preventing the contact of opposing shelves at equivalent concentrations.

Koshakji and Schultet (1973) gave a single subcutaneous injection of 380 mg/kg ASP or its hydrolysis products to rats on day 9 of pregnancy. ASP and SA induced a marked maternal body weight loss, a decrease in mean fetal weight, and increased incidences of fetal malformations and resorption, but SUA, 3DHB, and 5DHB exerted no significant toxic effects. When repeated oral doses of salicylates were given to pregnant rats, the minimal teratogenic dose was 150 mg/kg for both ASP (Tanaka et al., 1973; Wilson et al., 1977) and SA (Tanaka et al., 1973). This dosage is twice the human clinical dosage for ASP and 1.8 times that for SA.

By treating pregnant rats with repeated oral doses of ASP, Kimmel et al. (1971) demonstrated that only SA was present in both the maternal plasma and embryonic tissues and therefore SA might be the causative agent of ASP teratogenicity. The maximal concentration of SA in the maternal plasma following an oral administration of 150 mg/kg ASP to rats was 238 μg/ml (Wilson et al., 1977). This was similar to the maximal maternal serum level (221 μg/ml) after oral administration of 150 mg/kg SA (Tanaka et al., 1973). In our present in vitro study with fetal mouse palates, the minimal toxic concentration of SA was 0.25 mM (34.5 μg/ml), which was as low as 1/7 of the maximal SA level in the maternal blood following the SA administrations to rats cited above.

Rowland et al. (1972) performed a pharmacokinetic study on the absorption of ASP in humans, and confirmed the presence of ASP and SA in the plasma after ingestion of 650 mg ASP; the plasma level of ASP rose sharply to the maximal level of 23 μg/ml with a short biologic half-life of 14–20.5 min, while SA reached the maximal plasma level of about 50 μg/ml after ASP was hydrolyzed. Therefore, the minimal toxic concentration (2 mM, 360 μg/ml) of ASP to cultured fetal mouse palates was about 16 times the maximal plasma level of ASP in humans, but that of SA (0.25 mM, 35 μg/ml) was about 0.7 time the maximal plasma level of SA under human therapeutic conditions. The range of the therapeutic plasma concentrations of salicylates has been reported to be 50–100 μg/ml after treatment of analgetic diseases and 150–300 μg/ml after treatment of rheumatic diseases (Gabrielsson and Larsson, 1987). These concentrations were lower than the minimal toxic concentration of ASP but higher than that of SA observed in the present study with cultured fetal mouse palates. These data indicate that SA may be more toxic to developing embryos than ASP at comparable plasma concentrations. However, since the toxic plasma concentration of SA in humans is 200 μg/ml (Goodman and Gilman, 1985), its minimal toxic concentration to cultured mouse palates was lower than 1/5 of the toxic plasma concentration in humans.

Cultured rat embryos in the presence of 300–500 μg/ml ASP exhibited several types of malformations which were similar to those observed in vivo, i.e. curly tail, abnormal limbs, and craniofacial malformations (Yokoyama et al., 1984). Cicurel and Schmid (1986, 1988) demonstrated that the minimal concentration required for teratogenic effects was 150 μg/ml when rat embryos were exposed to ASP in vitro. Greenaway
et al. (1984) reported a result of in vitro exposure of rat embryos to sodium salicylate and 3 of its major metabolites at a concentration of 1.9 mM. Sodium salicylate, SUA, 3DHB, and 5DHB produced significant reductions in the crown-rump length and somite number, and sodium salicylate produced embryo lethality and also malformations in viable embryos. McGarrity et al. (1981) also showed that sodium salicylate adversely affects the growth of rat embryos at 300–800 μg/ml. When cultured rat embryos were exposed to 230 or 385 μg/ml SA, it induced malformations similar to those induced by ASP (Yokoyama et al., 1984).

In a micromass cell culture study, ASP caused 50% inhibition of the differentiation of rat embryonic midbrain cells at 165–190 μg/ml and that of limb bud cells at 89–190 μg/ml (Flint and Orton, 1984; Uphill et al., 1990). In an in vitro teratogen screening assay using human embryonic palatal mesenchyme cells, the concentration of ASP that inhibited the growth by 50% was as high as 755 μg/ml (Pratt and Willis, 1985). In our present study, the concentration of ASP that inhibited the fusion of cultured fetal mouse palates in 50% of the cases was 1.5 mM (278 μg/ml), which is within the range of toxic concentrations of ASP in other in vitro systems. The toxic concentrations in these in vitro studies were higher than the blood level of ASP under human therapeutic conditions, suggesting that the clinical doses of ASP may not be harmful to human embryos in utero. Since the toxic concentrations are generally not the same among different in vitro experimental systems, it is desirable to employ multiple in vitro testing systems and various toxicological indices when assessing teratological risks in the human based on in vitro data.

The degree of developmental toxicity to cultured fetal mouse palates was in a decreasing order of SA > ASP > SUA > 3DHB = 5DHB, which is similar to the data obtained in in vivo teratological studies and other in vitro systems. The susceptibility of cultured fetal mouse palates to salicylates was intermediate between the in vivo teratological susceptibility in the rat and the susceptibility of rat embryos cultured in vitro. The in vitro organ culture of fetal mouse palates might be useful for assessing the teratological potential of chemicals, especially those producing cleft palate, and could supplement in vivo teratologic experiments. The present study aimed at validation of palatal organ culture as a screening method of developmental toxicity, and detailed histologic studies of cultured palates are in progress in our laboratories.

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