Histopathological Findings of Cleft Palate in Rat Embryos Induced by Triamcinolone Acetonide

Satoshi FURUKAWA, Koji USUDA, Masayoshi ABE and Izumi OGAWA

1) Biological Research Laboratories, Nissan Chemical Industries, Ltd., 1470 Shiraoka, Minamisaitama Saitama 349–0294, Japan

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ABSTRACT. Triamcinolone acetonide (TAC), a synthetic glucocorticoid, induces cleft palate resulting from poor development of palatal shelves in mice. However, TAC has no effect on medial edge epithelial cells (MEE cells) in secondary palatal shelves. In the present study, we examined the relationship between the pathogenesis of cleft palate and the effects on MEE cells and palatal mesenchymal cells in rat embryos/fetuses exposed to TAC. Pregnant Wistar Hannover rats were given TAC intramuscularly at 0.5 mg/kg at gestation days (Day) 12, 13, and 14, then embryos/fetuses were harvested on Days 14.5, 15, 16 and 20. The effects of TAC were as follows; an inhibition of palatal mesenchymal cell proliferation on Day 14.5, a decrease in the density of palatal mesenchymal cells and MEE cells, and expression of epidermal growth factor (EGF) receptors in MEE cells on Day 15, and stratified squamous differentiation of MEE cells with expression of cytokeratin and EGF receptors on Day 16. These findings indicated that TAC inhibited the proliferation of mesenchymal cells and affected the differentiation of MEE cells into stratified squamous epithelia in the palatal shelves of rat embryos. However, these stratified squamous MEE cells partially fused with each other. Thus, we suspected that a major contributing factor to the formation of TAC-induced cleft palate might not be the altered differentiation of MEE cells, but the inhibition of mesenchymal cell proliferation.

KEY WORDS: cleft palate, MEE cell, rat, triamcinolone acetonide, Wistar Hannover.

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Cleft palate is one of the most common malformations in laboratory animals under various experimental conditions, including irradiation and the presence of various chemicals [15]. It may result from disturbances at any stage of palatal development: defective palatal shelf growth, delayed or failed shelf elevation, defective shelf fusion, failure of medial edge epithelial cell (MEE cell) death, postfusion rupture and failure of mesenchymal consolidation and differentiation [10]. Numerous reports have described the effects of glucocorticoids [19, 21] or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [6, 8] on the developing fetus through administration during mammalian gestation. Both TCDD and glucocorticoids induce cleft palate by acting through aryl hydrocarbon receptor- and glucocorticoid receptor-mediated mechanisms, respectively [1, 4]. In addition, the different effects on MEE cell proliferation and differentiation are suggested in TCDD- and glucocorticoid-induced cleft palate in mice, morphologically [17]. On the other hand, there are few reports describing the histopathological changes of the glucocorticoid-induced cleft palate in rats [7, 14, 16].

In order to investigate the effects of glucocorticoids on secondary palatal shelves in cleft palate in rats, we examined the histopathological and immunohistochemical changes of MEE cells and palatal mesenchymal cells in rat embryos/fetuses prior to or during fusion in the presence of triamcinolone acetonide (TAC) [20], a synthetic glucocorticoid.

MATERIALS AND METHODS

Animals: A total of 32 pregnant specific pathogen-free Wistar Hannover GALAS rats (CLEA Japan, Inc.), at approximately 9 weeks of age, were purchased. The animals were single-housed in wire-mesh cages in an air-conditioned room (22 ± 2°C; humidity, 55 ± 10%; light cycle, 12 hr/day). Feed (Oriental Yeast Co., Ltd., Japan) and water were available ad libitum.

Experimental design: Gestation day (Day) 0 was designated as the day when the presence of vaginal plugs was identified. The animals were injected daily with a single intramuscular dose of 0.5 mg/kg BW of TAC dissolved in a 0.9% NaCl solution during Days 12–14. This dose and the exposure period of TAC were previously found to produce cleft palate in 100% of rat fetuses [18]. An equal volume of 0.9% NaCl solution was administered to the animals in control group. All injections were made between 10 and 11 a.m. Four rats from each group were sampled on Days 14.5, 15, 16, or 20. Animals were weighed, euthanized by exsanguination under diethyl ether anesthesia, and necropsied. The live embryos/fetuses were removed from the uterus, weighed, and examined for external malformation with the aid of a dissecting microscope. These experiments were conducted according to the Guideline for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

Histological examination: The embryos/fetuses were fixed in 10% neutral buffered formalin. Two or five embryos/fetuses were randomly selected from each litter, embedded in paraffin, sectioned at a 4-μm thickness, and stained routinely with hematoxylin and eosin (HE) for histopathological evaluation. Immunohistochemical staining involving proliferating cell nuclear antigen (PCNA) (PCNA, PC10: DAKO, Japan), cytokeratin (Cytokeratin, MNF116: DAKO, Japan), epidermal growth factor (EGF) receptors (EGFR, 1005 SC-03: Santa Cruz Biotechnology,
Inc., U.S.A.), and in situ TUNEL (Apoptosis in situ Detection Kit Wako: Wako Pure Chemical Industries, Japan) were performed according to the avidin-biotin complex (ABC) method (VECTStain ABC Kit: Vector Laboratories Inc., Canada). For PCNA labeling index (LI%) on Days 14.5 and 15, labeled nuclei and total nuclei were scored in one section for palatal mesenchymal cells, MEE cells, nasal epithelia, and oral epithelia with a \( \times 50 \) objective. LI% of individual animals was determined by dividing the total number of PCNA-labeled cells by the total number of cells counted by light microscopy with the aid of an image analyzer (IPAP; Image Processor for Analytical Pathology: Sumika Techno Service Co., Japan). LI% on Days 16 and 20 were not counted because the shape of the palatal shelves was different between fused control and unfused treatment specimens.

**Statistical analysis:** Means and standard error (SE) were calculated. Continuous data were analyzed with the F tests. When variances were homogeneous, the Student's \( t \)-test was performed. When significant, an Aspin-Welch test was employed. Statistical difference from control values for quantitative data was determined using the Wilcoxon rank sum test.

### RESULTS

Table 1 shows the numbers and weights of examined live embryos/fetuses and the incidence of open palate at each sampling time point. Statistically significant decreases in treated fetal weights were seen on Day 20. For most control embryos, fusion of the shelves was completed by Day 16 and there was no cleft palate incidence on Day 20, whereas cleft palate was induced in 100% of treated fetuses.

**Day 14.5 of gestation:** As shown in Fig. 1, a significant decrease in LI% of the mesenchymal cells was observed in treated embryos. There were no other remarkable morphological differences between control and treated palatal shelves. At this stage the MEE cells in both groups were negative for cytokeratin and EGF receptors. The epithelia and mesenchymal cells contained no TUNEL-positive nuclei in both groups.

**Day 15 of gestation:** In control embryos, the epithelia of the palatal shelves were composed of double cell layers (Fig. 2a). The cells of the basal layer and the superficial layer were cuboidal or columnar, and flat-shaped, respectively. They showed a gradual transition from columnar to cuboidal-shaped in the medial to lateral direction. In treated embryos, the MEE cells formed single or double cell layered epithelium, which were flat, cuboidal, or irregular in shape (Fig. 2b). Although there were no significant differences in LI% of the MEE cells and the mesenchymal cells between control and treated palatal shelves, decreased cellular density of the MEE and mesenchymal cells in the palatal shelves was seen in treated group. The MEE cells were negative for cytokeratin and positive for EGF receptors in treated group. The epithelia and mesenchymal cells contained no TUNEL-positive nuclei in both groups.

**Day 16 of gestation:** The control palatal shelves were in contact, and fusion was in progress in most of the embryos. Disappearance of the MEE cells, and the formations of midline epithelial seams and the epithelial triangle were observed along with apoptotic bodies (Fig. 3a). In control embryos in which palatal fusion had not occurred, the MEE cells were stratified columnar or cuboidal in shape (Fig. 3b). The epithelial seams and the MEE cells were negative for cytokeratin (Figs. 3a, 3b) and negative or slightly positive for EGF receptors (Fig. 3c). In treated embryos, although the palatal shelves were elevated from vertical to horizontal positions, palatal fusion did not occur. The MEE cells differentiated into stratified squamous cells which were positive for cytokeratin and EGF receptors, at the unfused (Figs. 3d, 3e) and partial fused portions (Fig. 3f). As in Day 15, decreased cellular densities of the MEE cells at the portions around the squamous epithelia, and the mesenchymal cells were noted. TUNEL-positive nuclei were observed in the midline epithelial seams and epithelial triangle. The mesenchymal cells contained no TUNEL-positive nuclei in both groups.

**Day 20 of gestation:** In control fetuses, the two palatal

<table>
<thead>
<tr>
<th>Gestation day</th>
<th>Treatment</th>
<th>No. of pregnant animals</th>
<th>Total No. of live embryos/fetuses</th>
<th>Embryo/fetus weight (g)</th>
<th>Incidence of open palate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5</td>
<td>Control</td>
<td>4</td>
<td>40</td>
<td>0.19 ± 0.02</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>TAC</td>
<td>4</td>
<td>44</td>
<td>0.14 ± 0.00</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td>15</td>
<td>Control</td>
<td>4</td>
<td>30</td>
<td>0.28 ± 0.04</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>TAC</td>
<td>4</td>
<td>28</td>
<td>0.33 ± 0.04</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td>16</td>
<td>Control</td>
<td>4</td>
<td>43</td>
<td>0.71 ± 0.01</td>
<td>21.0 ± 12.20</td>
</tr>
<tr>
<td></td>
<td>TAC</td>
<td>4</td>
<td>38</td>
<td>0.57 ± 0.05</td>
<td>100.0 ± 0.00*</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>4</td>
<td>51</td>
<td>3.27 ± 0.06</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>TAC</td>
<td>4</td>
<td>9</td>
<td>1.63 ± 0.02*</td>
<td>100.0 ± 0.00*</td>
</tr>
</tbody>
</table>

Mean ± SE.

*: Significantly different from control at \( p<0.05 \) (Wilcoxon rank sum test).

**S. FURUKAWA, K. USUDA, M. ABE AND I. OGAWA**
shelves fused and the midline epithelial seams disappeared. The epithelia on the nasal aspect of the palate differentiated into pseudostratified ciliated columnar cells, while those on the oral aspect differentiated into cytokeratin-positive stratified squamous cells (Fig. 4a). In treated fetuses, the epithelium covering the palatal shelves showed a gradual transition from oral stratified squamous epithelium to nasal respiratory epithelium at the position of the MEE cells. The stratified squamous epithelia and a few nasal respiratory epithelia were positive for cytokeratin (Fig. 4b). At this stage the epithelia on the nasal aspect and oral aspect were positive for EGF receptors, and the epithelia and mesenchymal cells contained no TUNEL-positive nuclei in both groups.

**DISCUSSION**

We have shown that TAC inhibited the proliferation of mesenchymal cells and affected the differentiation of MEE cells into stratified squamous epithelia in the palatal shelves of rat embryos. However, a major contributing factor to the formation of TAC-induced cleft palate might not be the altered differentiation of the MEE cells, but the inhibition of mesenchymal cell proliferation.

The inhibitory effect of glucocorticoids on cell proliferation is documented in various systems [12]. Glucocorticoids inhibit RNA synthesis in the palatal shelves [5] or induce the formation of small palatal shelves due to a decrease in mitotic density [4, 9, 12]. Kurisu et al. observed
TAC induced retardation of the elevation of palatal shelves, and degradation of MEE cells within a half or one day [13]. Thus, the major factor contributing to glucocorticoid-induced cleft palate in mice is the failure of palatal shelf to elevate and make extensive contact. Our results showed that TAC inhibited mesenchymal cell proliferation in the palatal shelves at the stage of development. Therefore, in TAC-induced cleft palate in rats, the inhibition of mesenchymal cell proliferation is considered to be a major contributing factor towards poor development of palatal shelves, as in mice.

In the development of mammalian palatal shelves, the nasal epithelia differentiate into pseudostratified ciliated columnar cells and the oral epithelia differentiate into stratified squamous cells. On the other hand, MEE cells form epithelial seam and disappear by apoptosis and cell migration into the mesenchymal cells involving an epithelial-mesenchymal transformation [10, 11, 22]. In TCDD-induced cleft palate in mice, the differentiation of MEE cells is altered along with expression of EGF receptors [2]. In rats, altered differentiation of MEE cells is examined by organ culture, since TCDD is maternally toxic rather than a cause of malformations [3]. Our results indicated that the MEE cells in TAC-treated embryos exhibited squamous differentiation along with expression of cytokertatin and EGF receptors. These morphological findings in the MEE cells resembled features in TCDD-induced cleft palate in mice and rats. Consequently, we showed that TAC induced altered differentiation in MEE cells in rats, similar to TCDD-induced cleft palate.

Abbott et al. reported that altered differentiation of MEE cells prevented fusion of the shelves, resulting in cleft palate in TCDD-treated animals [1-4]. However, Takagi et al. demonstrated that the formation of TCDD-induced cleft palate was not related to altered differentiation of MEE cells, but to the poor development of palatal shelves, since exencephalia inhibited TCDD-induced cleft palate in mice [23]. In our study, the stratified squamous MEE cells contacted each other and fusing epithelial seams were partially observed. This suggested that the altered differentiated MEE cells might still have the ability to contact and fuse with each other. In addition, the effects of TAC on MEE cells may have not contributed to the induction of cleft palate in mice, since TAC does not prevent degeneration or cessation of DNA synthesis in MEE cells [4, 13]. Therefore, we suspected that altered differentiation might not be related to the formation of TAC-induced cleft palate in rats.

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REFERENCES


Fig. 3. Palatal shelves at gestation day 16. Bar=50 µm/500 µm (Inset) a) Control. In fused palatal shelves, the epithelial triangle (↑) are negative for cytokeratin. b) Control. In unfused palatal shelves, MEE cells are negative for cytokeratin. c) Control. In fused palatal shelves, the epithelial triangle (↑) are negative for EGF receptors. d) TAC-treated. Stratified squamous MEE cells are positive for cytokeratin. e) TAC-treated. MEE cells are positive for EGF receptors. f) TAC-treated. At fused portion, the epithelial triangle (↑) are positive for cytokeratin.

Fig. 4. Palatal shelves at gestation day 20. Bar=50 µm/500 µm (Inset) a) Control. The stratified squamous keratinizing cells on the oral aspect are positive for cytokeratin. b) TAC-treated. The stratified squamous epithelia on the oral aspect and a few nasal respitory epithelia are positive for cytokeratin.


