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Retinoic Acid Induces Abnormal Palate During Embryogenesis in Rat

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In order to understand the effects of all-trans-RA on palate development, RA was injected into the abdominal cavity of pregnant mice and then the embryos were taken in the following days and analyzed morphologically as well as molecular biologically. When RA was administered at the stage of E11 or E15, the overall craniofacial development was retarded. The length from jaw to eye was shortened, compared to that of normal group. When the E11 embryos were exposed to RA, cleft lip was also found along with the cleft palate. *In vitro* palate culture experiment also revealed that RA caused cleft palate. When RT-PCR was performed, early stage administration of RA at E11 inhibited the upregulation of *Hoxa7* expression at E15 through E17. Whereas in control group, high level of *Hoxa7* expression was detected in the palate of E15 to E17. In the case of *Bax*, the expression was decreased from E16, while remaining constant in control group. When TUNEL analysis was performed following the RA treatment at E15, TUNEL positive cells were detected in the mesenchymal cells as well as epithelial cells of palatal shelves of E16 and in E17 embryos. Whereas in normal control, TUNEL positive cells were observed mostly at the epithelium around the nasal cavity and oral cavity where rugae is made. These results altogether indicate that exposure to RA during palate development causes facial deformity including cleft palate and cleft lip by modulating the expression of homeotic genes such as *Hoxa7* as well as an apoptosis-related gene, Bax, and thus malregulating the apoptosis.

Key Words: Palatogenesis, Cleft palate, Retinoic acid, *Hoxa7*, *Bax*, Apoptosis

INTRODUCTION

Palate of mammals prevents mixing between the paths of food and air by separating nasopharynx and oropharynx and enables the baby to suck breast by forming vacuum in the oral cavity, and it is also an important organ for pronunciation of language. Development of palate in humans was divided into two steps, the primary and secondary palate. This developmental process begins from the early 6th week of pregnancy. The important period of palate formation is between late 6th week and early 9th week. The mechanism of palatogenesis involves the formation of primary palate, which leads to formation of the front and middle part of maxilla. The process of primary palate takes place with the development of medial palatal prominence through incorporation of medial nasal prominences between the interior surfaces of the maxilla. Secondary palate consists of hard part with rugae (front) and soft part (back). The process of formation of secondary palate starts with the expansion of mesenchymal prominences from maxillary prominence in early 6th week of development and also passing through the 7th and 8th weeks of development. Palatal shelves develop at both sides of tongue protrude downward. At the end of 8th week, the palatal shelves heave up above the tongue for moment and maintain horizontal balance (Keith et al., 208). The palatal shelves of both sides start incorporation at the right center by approaching each other. Both sides of the medial edge epithelia (MEE) at palatal shelve tip contact each other and form midline seam. Finally the complete incorporation occurs as MEE disappear and basal membrane collapses

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(Ferguson, 1987).

To understand the developmental process of palatogenesis, rat has been extensively used as an animal model, since rat palatogenesis is mostly similar to that of human. In rat, palatal shelves start to grow in E14 and E15 with the downward movement. In E16, palatal shelves start to grow upward above the tongue and maintain balance of horizontal position, and then finally center around and contact each other. The complete palate is formed by E17 (Srivastava and Rao, 1979).

Cleft palate is the most widespread congenital facial deformity occurring in 1 or 2 human beings out of 1,000. This occurs more frequently to women than men (Kim et al., 2002; Marazita et al., 2002). Cleft palate is formed due to growth delay of palatal shelves itself, delay or failure of horizontal array of palatal shelves that are growing vertically for the first time, or contact abnormality of both sides of palate shelves after horizontal array. Cleft palate is also due to the abnormal cell death of MEE, secondary destruction after incorporation, abnormal condensation and differentiation of mesenchymal cells around palate. Furthermore, hereditary and/or environmental factors are also known to be involved. Among the environmental factors, excessive or shortage of certain vitamin, hormone or drug have been reported to induce cleft palate during embryogenesis (Ferguson, 1987).

All-trans retinoic acid (RA) is an endogenous metabolite of vitamin A, which is known to be necessary substance for normal embryo formation during animal development (Morriss-Kay, 1992; Brickell and Thorogood, 1997; Yu et al., 2003). It has been widely used for treatment of pimple and other skin infections etc. It is reported that excessive or insufficient RA causes craniofacial deformity, thymic nonformation, heart outflow tract defect and neural tube defect including cleft palate at the early stage of pregnancy (Brickell and Thorogood, 1997). Endogenous RA plays an important role in apoptosis of MEE during fusion of palatal shelves (Cuervo et al., 2002). Several reports suggested that exogenous and/or excess RA can act as a strong deformity causing substance (Abbott et al., 1989; Newall and Edwards, 1981a; Newall and Edwards, 1981b; Sulik et al., 1988).

Hox genes encode transcription factors that are known to be involved in formation of embryo by expressing at particular time and particular area during embryo development (Kaiser et al., 2003). They are known to affect embryogenesis under the influence by RA (Kessel and Gruss, 1991; Marshall et al., 1996). When the RA is introduced during vertebrate development homeotic transformations have been induced at the cervical spine, chest, waist and sacral vertebra, etc., due to the changes in *Hox* gene expression. Ectopic expression of *Hoxa7*, *Hoxb6*, *Hoxb7*, *Hoxd4* has been reported to induce craniofacial malformation including cleft palate (Whiting, 1997; Balling et al., 1989; Mclain et al., 1992; Lufkin et al., 1992). Interestingly enough, the morphological changes induced by the ectopic expression of *Hox* genes were similar to those exposed to RA.

In this study, we compared and analyzed the process of cleft palate formation by morphological observations at different stages of development following the injection of RA. We also examined the effect of RA in palatal tissues cultured *in vitro*. To understand the underlying molecular mechanism during cleft palate formation, *Hox* as well as apoptosis related gene was analyzed in RA treated embryos, along with TUNEL staining.

MATERIALS AND METHODS

Animal management and RA treatment

Rat strain Spargue-Dawley (SD) was used in this study. Adult SD rats were maintained at room temperature under dark/light cycle of 12/12. The rats were mated and the presence of a vaginal smear was defined as embryonic day 0. Pregnant rats were injected intraperitoneally with 100 mg/kg all-trans RA (R-2625, sigma, St. Louis, Missouri, USA) dissolved in sesame oil (S-3547, Sigma, St. Louis, Missouri, USA) at E11 or E15 stage. In control group, sesame oil including DMSO was injected.

Dissection of embryos and morphological observation

E13 and 17 embryos were collected from both control and RA injected group by using standard ethical animal procedures. The collected embryos were dipped in autoclaved PBS and the mandible regions were removed under the optical microscope (M10, Leica, Switzerland). The

palate samples were collected following the standard methods (Cheng et al., 2006) for different experimental purposes. In order to perform the immunohistochemistry, the maxillary region was dissected and fixed in 4% para-formaldehyde (pH 7.4).

Total RNA isolation and RT-PCR

For isolation of total RNA, RNA Zol B (LPS industries inc., New Jersey, NY, USA) was used. Complementary DNA (cDNA) was prepared by reverse transcriptase (RT) using 2 μg of total RNA in a reaction volume of 25 μl. And then 1 μl of cDNA was used for the subsequent PCR using rat *Hoxa7*, *Bax* and β-*actin* specific primers sets. Beta actin was used as an internal control. PCR reaction conditions were dependent upon the primer sequences (see text). PCR products were loaded onto the 1.5% agarose gel, visualized under UV light, and then photographed with Dolphin-DOC (Wealtec, Sparks, NV, USA).

***In vitro* organ culture**

Palatal shelves were separated from E15.5 embryos and cultured on 1 μm Millipore filter membrane and a metal grid in a falcon organ culture dish with serum free DMEM/F12 (Welgene, Daegu, Korea) containing 20 μg/ml ascorbic acid (A0278, sigma, St. Louis, Missouri, USA) and 1% penicillin/streptomycin, and then the medium was changed every 24 hours. RA was used in culture medium at 20 μM final concentration. The culture dish was placed in a 5% CO_2 and 37℃ humidified incubator for 60 hours. Tissues were then fixed and processed for hematoxylin-eosin (H-E) staining.

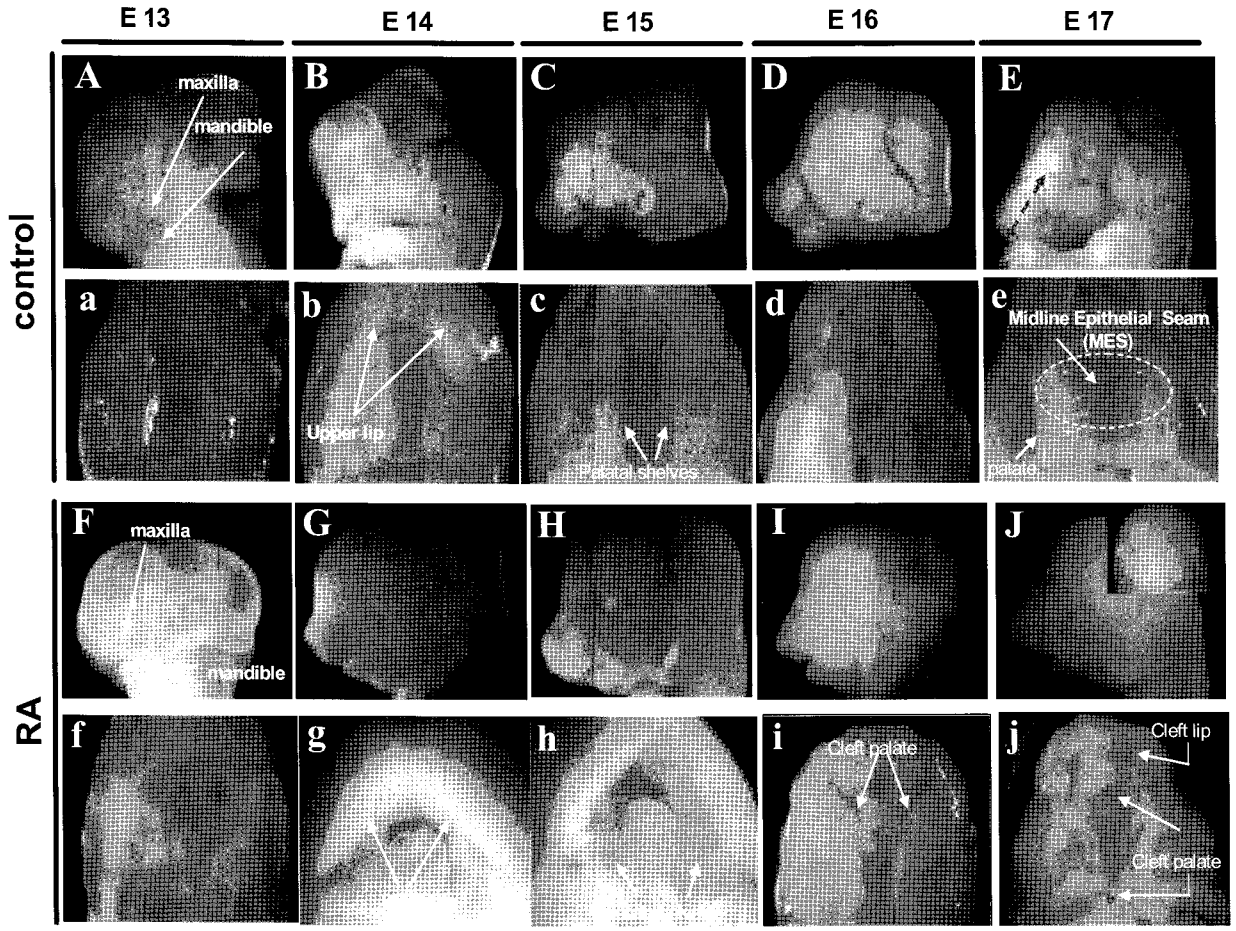


Fig. 1. The effect of RA on the morphology of developing head and palate. Retinoic acid was injected into the abdominal cavity of rat at embryonic day 11. The craniofacial **(A-J)** as well as palate **(a-j)** morphology of control **(A-E** and **a-e)** and RA-treated embryos **(F-J** and **f-j)** are shown.

Immunohistochemistry and TUNEL assay

After fixation, the tissues were embedded in OCT compound (Triangle Biomedical Science, Durham, NC, USA). Using microtome, tissue sections (12 μm thickness) were prepared and then incubated at 4℃ overnight with the primary goat polyclonal antibody against *Hoxa7* (sc-17152, Santa Cruz, Delaware, CA, USA). All Slides were washed with PBS, the specimens were allowed to react with biotinylated rabbit antigoat immunoglobulins as a secondary antibody and streptavidin peroxidase at room temperature for 10 minutes. The specimens were visualized using a 3, 3'-diaminobenzidine (DAB) reagent kit (Zymed, South San Francisco, CA, USA).

In order to analyze the apoptosis, TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay was performed using *in situ* cell death detection kit (Roche, Mannheim, Germany). The 12 μm thick sections were treated with 0.1% triton x-100 in sodium citrate for 2 minutes on ice. The samples were incubated with the labeling reaction mixture at 37℃ for 1 hour. The slides were observed under the fluorescent microscope (Olympus 1×70, Olympus corp., Melille, NY, USA).

RESULTS

***In vivo* effect of retinoic acid on craniofacial and palate development**

In order to study the effect of RA on the morphology of embryos during palatal development, the embryos from control and RA treated groups were compared. The RA was injected at the stage of E11 (early stage of palate development), and then E13 through E17 embryos were isolated and the morphology was analyzed under optical microscope. Developments progressed both in control group and RA treated group, however RA treated group showed retardation of overall craniofacial development. In addition, the length from jaw to eye was shortened (Fig. 1J red arrow) compared to that of the control group (Fig. 1E). Also the eye development seemed to be delayed in RA treated group (Fig. 1I, J, D and E). When we analyzed the

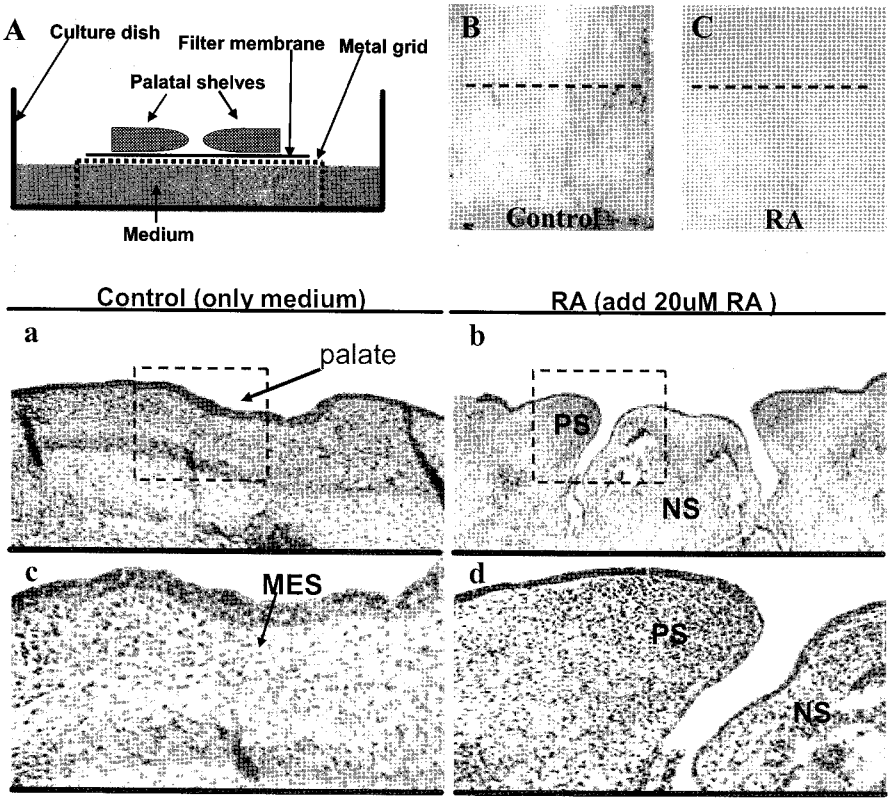


Fig. 2. *In vitro* effect of RA on the morphology of palatal shelves Schematic of *in vitro* organ culture system **(A)**, The palate of control group **(B, a, c)** and RA-treated group was culture for **(C, b, d)** was seen in the photograph. PS: palatal shelves, MES: midline epithelium seam, NS: nasal septum. Blue line: 1 μm filter membrane, The black dotted line is sectioning direction.

palate morphology after removing mandible, the medial edge epithelia (MEE) of palatal shelf tips contacted each other and fused completely in E16 and E17 embryos (Fig. 1d, e) by forming MES in the case of control group, whereas RA-treated group showed no contact of MEE. (Fig. 1i), and sometimes delayed fusion of palate with partially formed MES and cleft lip as well were also detected (Fig. 1j).

In vitro effect of retinoic acid on palate formation through organ culture

To see the *in vitro* effect of RA on palate formation, *in vitro* organ culture was applied here (Fig. 2A). The palate was dissected out at the stage of E15.5 and cultured for 60 hours in the presence or absence of RA (20 μM) to reach the *in vivo* stage of E17, and then compared the palatal shelves after sectioning and H-E staining. As shown in Fig. 2, the palate of control group has been fused (Fig. 2B, 2a & 2c), whereas RA treated group showed no fusion of palate (Fig. 2C, 2b & 2d). This result further proved that the *in vivo* and *in vitro* effect of RA on palate formation are almost similar each other.

Effect of RA during the late stage of palatogenesis

Since organ culture experiment indicated that the administration of RA at the late stage of palatogenesis (E15) still caused the malformation of palate, RA was injected at the stage of E15, and then E17 embryos were harvested and analyzed their morphology. As shown in Fig. 3, no palate fusion was observed in RA group (Fig. 3B, a', b', and c'), whereas control group showed normal palate formation (Fig. 3A, a, b, and c). These results also indicate that RA causes severe effect on palatogenesis during embryo development irrespective of the day of injection.

Effect of RA on the homeotic as well as apoptosis-related gene expression during palatogenesis

To find out whether the homeotic and apoptosis related gene expressions were modified by RA administration, total RNAs were isolated from palate of E13 to E17 embryos, and then RT-PCR was performed with the gene specific primers listed in materials and methods. *Hoxa7* expression was increased from gestation day 15, where the palatal shelves grow vertically and remained constant until E17. On the other hand, *Hoxa7* expression was not increased throughout the stage E17 in the embryos exposed to RA (Fig. 4). In the case of *Bax*, the expression levels were almost similar from gestation day of 13 to 15, but a dramatic decrease in *Bax* expression was observed in RA treated groups in E16 and E17 (Fig. 4).

*Hox*a7 expression pattern during palatogenesis following RA treatment: immunohistochemistry

To analyze the *Hoxa7* expression in palate, embryos of E16 and E17 were collected after injection of RA on gestation day of 15, and then the immunohistochemistry was performed with *Hoxa7* antibody (Fig. 5). Control group

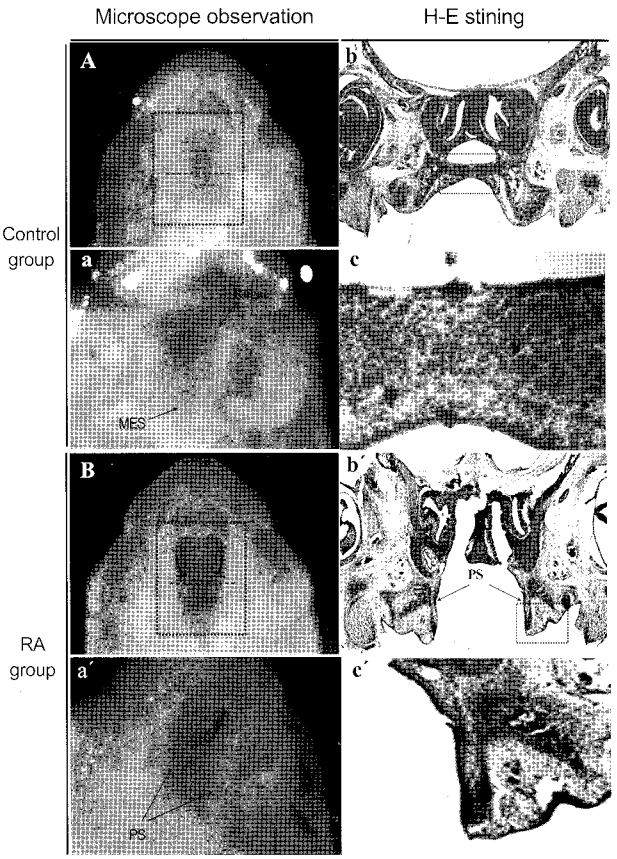
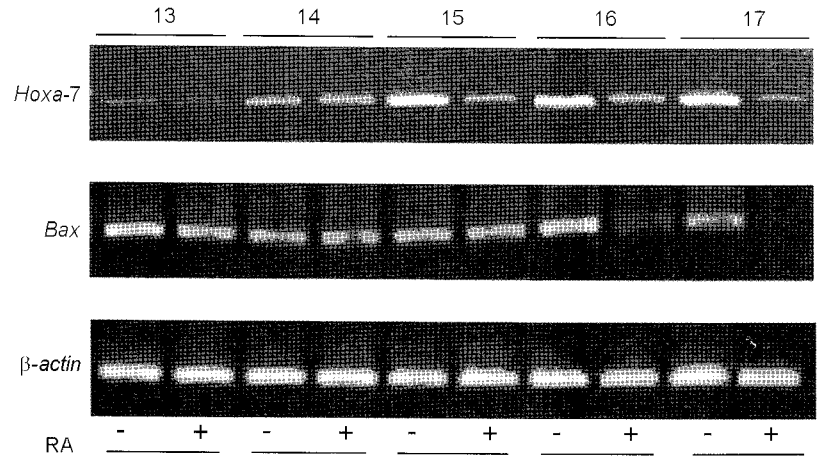
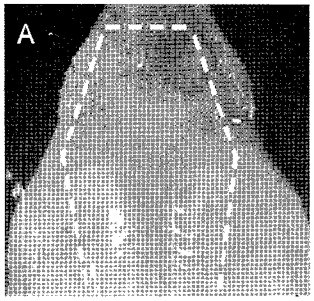


Fig. 3. Different shape of closure of palatal shelves in embryo of control and RA treated group (RA injected at the embryonic stage of E15). The morphology of E17 palate following the injection of RA on gestation day E15. Control group, palatal shelves were completely fused **(A, a, b, c)** whereas RA group, cleft palate was formed **(B, a', b', c')**. PS: palatal shelves, MES: medline epithelial seam





RT-PCR conditions

Primer	Product size	Sequence	Annealing temp. / cycle
Hoxa-7	425 bp	5'-CCCAAGCTGGTCAAAATT-3' 5'-GATATCCTGGCCCTTTACTC-3'	56℃/28
Bax	496 bp	5'-GCTCTGAACAGATCATAAG-3' 5'-GCCACTCCTGCTCGAAG-3'	50℃/30
β-*actin*	327 bp	5'-CATGTTTGAGACCTTCAACACCCC-3' 5'-GCCATCTCCTGCTCGAAGTCTAG-3'	50℃/28

Day	Control	RA
13	8 embryos	9 embryos
14	10 embryos	8 embryos
15	10 embryos	11 embryos
16	10 embryos	9 embryos
17	7 embryos	14 embryos

Fig. 4. Semi-quantitative expression analysis of *Hoxa7* and *Bax* genes in control and RA group during palate development. The palate tissues (A) only were collected from each stage (E13, 14, 15, 16, & 17) of embryos following the injection of RA (+) or control (−) at the embryonic stage E11. Total numbers of embryos used for synthesizing cDNAs are indicated in the box below right. RT-PCR was performed with appropriated primers and conditions written in the box below left.

of E16 has Hoxa7 expression on both sides of palate (Fig. 5A, a), not where MEE is conjugated, whereas E17 showed the expression at the central part of palate where midline seam is formed (Fig. 5B, b). In the case of RA injected group, low amount of *Hoxa7* expression was detected (Fig. 5C, c, D, and d).

Effect of RA on apoptosis in developing palate

To analyze the effect of RA on apoptosis during palatogenesis, TUNEL staining was performed in the embryos (E16 and E17) treated or non-treated with RA at the embryonic stage of E15. As shown in Fig. 6, both sides of MEE of palatal shelves contacted each other in E16 control group, and the TUNEL positive cells were observed mostly at the epithelium surrounding nasal cavity and oral cavity which forms rugae, whereas E17 when palatal incorporation is nearly reached, TUNEL positive cells were observed at the region where MES disappears (Fig. 6A, B). In the case of RA treated group, TUNEL positive cells were distributed in the mesenchymal cells and epithelial cells of palatal shelves in E16 embryos. In E17, apoptotic cells were distributed in the areas immediately below the epithelium of palatal shelves (Fig. 6C, D).

DISCUSSION

In order to understand the effects of all-trans-RA on palate development, RA was injected into the abdominal cavity on the 11^{th} or 15^{th} day of pregnancy, and then the embryos in the following days were taken and analyzed morphologically as well as molecular biologically.

Morphological analysis showed that the embryos exposed to RA harbored cleft palate in which palatal shelves fusion did not occur (Fig. 1i, j). In the case of normal embryo,

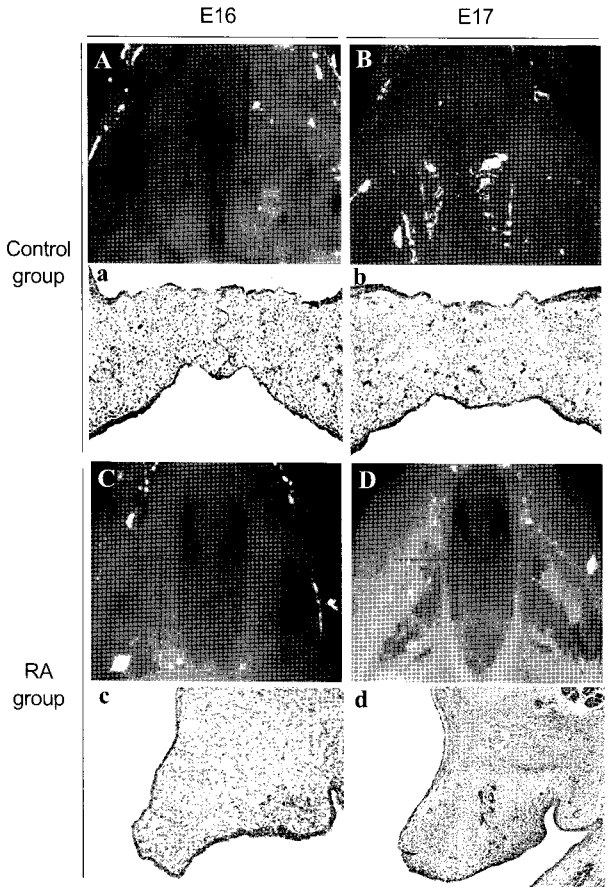


Fig. 5. Immunohistochemistry using *Hoxa7* antibody on developing palate. On the palate in control group E16 (A, a), expression of *Hoxa7* was shown mostly at both sides of palate, and on E17 (B, b), expression was shown mostly mesenchymal cells at central part of palate that forms midline seam. However, the palate of embryos to which RA is infused, expression was in small amount compared to control group at mesenchymal cell (C, D, c, d)

palatal shelves were elevated at the stage of E15 (Fig. 1c), contact and fusion of palatal shelves started in E16 (Fig. 1d) forming complete palate in E17 (Fig. 1e). Interesting thing is that embryo with cleft palate and/or cleft lip was observed when RA is infused in E11, but the group to which RA is infused in E15 showed only cleft palate (Fig. 3B, a', b', c'). Such cleft lip and cleft palate occur while this process is delayed by RA in E11, when the maxillary prominence forms outside upper border of stomatodeum by growing toward the center and being separated by groove from lateral nasal prominence and nasal pit starts to be visible from outside of embryo. However if RA was injected in E15, when the two palatal shelves were projected from inside maxillary prominence after this process completes, cleft palate was observed (Fig. 3). *In vitro* organ culture experiment also exhibited that RA treatment prohibited the development of palatal shelves, which resulted in the open palatal shelves (Fig. 2). We also attempted to analyze the palate morphology after birth following the infusion of RA at the concentration of 100 mg/kg at both stage of E11 and E15. In both cases, however, embryo could not come into birth and this could be due to the concentration of RA (100 mg/kg), which is a lethal concentration making the birth of embryo hard.

In order to understand the molecular mechanism underlying the palatogenesis, *Hox* as well as apoptosis related gene expression pattern was analyzed with the dissected palate. The embryos injected with RA (100 mg/kg) at the developmental stage of E11 or E15 were dissected out in the following days (E13 to E17), and then the palate was isolated. After purifying total RNA, cDNA was synthesized, and then RT-PCR was performed with *Hoxa7* as well as apoptosis-related gene specific primer sets. When RA was injected in E11, expression of *Hoxa7* was increased in E15 and this increased expression pattern remained constant until E17. However in RA treated group, the *Hoxa7* expression level was not changed until E17 (Fig. 4).

Immunohistochemistry analysis has revealed that *Hoxa7* expression in E16 and E17 was reduced in the experimental group compared to that normal groups. These results implies that *Hoxa7* protein, probably plays an important role during contact and fusion of palatal shelves (Fig. 5).

In general, endogenous RA has been reported to play an important role during programmed cell death occurred in MEE during palatal shelf fusion. However, exogenous RA can act as a strong teratogen depending on the dosage as well as development stages during palatogenesis (Abbott et al., 1989; Newall and Edwards, 1981a; Newall and Edwards, 1981b). It was reported that in pregnant mouse, apoptosis was induced in various cells when RA was treated. In previous study performed with total head RNA, *Bax* gene expressed consistently at high level form E13 to E17. Whereas in RA treated group, the expression pattern of *Bax* gene was gradually increased from developmental stage of E15 throughout E17 (Nazarali et al., 2000). In this study, however, *Bax* expression was reduced when RA was

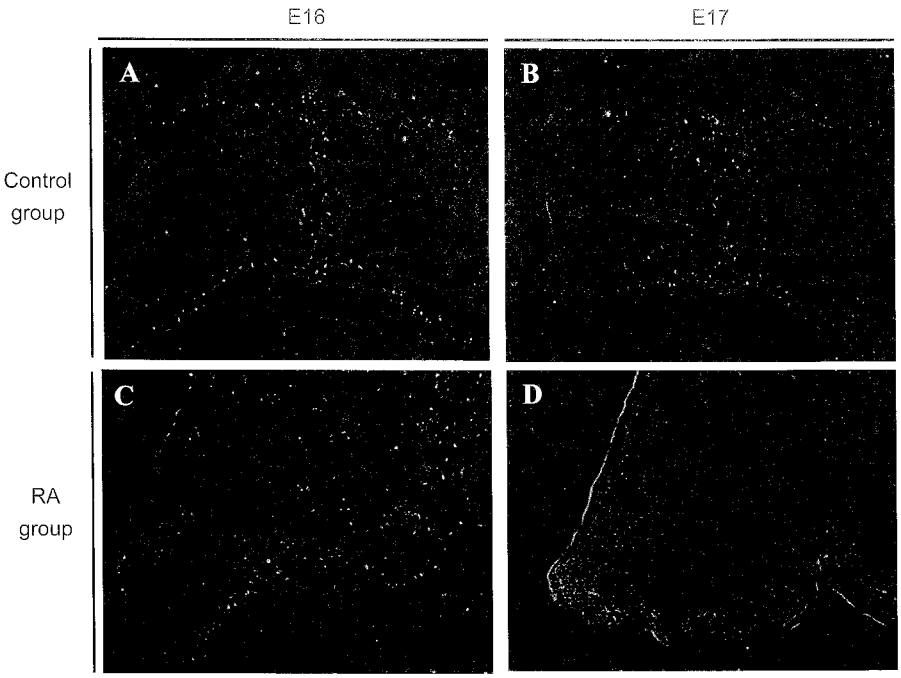


Fig. 6. Apoptosis patterns during palatogenesis by TUNEL analysis. **A, B:** palatal shelves in control group, **C, D:** palatal shelves in RA treated group

treated in E11 (Fig. 4). TUNEL assay also showed that RA treatment inhibited apoptosis (Fig. 6). In the case of control group, TUNEL assay revealed that the TUNEL positive cells were mostly observed at the region of MES, the epithelial cells surrounding nasal cavity as well as oral cavity where rugae is made in E16 (Fig. 6A). In E17, TUNEL positive cells were observed at the region where MES is incorporated (Fig. 6B). In the case of RA treated group, TUNEL positive cells were found in both epithelial and mesenchymal cells in palatal shelves at the stage of E16 (Fig. 6C), while E17 palate has shown TUNEL positive cells only in or near epithelial cells of palatal shelves (Fig. 6D). Previously, it has been reported that the apoptosis is required for palate formation, especially at the marginal MEE during palate development (Martinez et al., 2000; Gurley et al., 2004). If apoptosis does not occur properly, cleft palate can be formed because complete fusion is not accomplished although palatal shelves may go through conjugation. The results here indicate that the RA treatment inhibited apoptosis whereas previous result strongly implied the induction of apoptosis during palatogenesis. The discrepancy could be explained partially by the dosage of RA administered at the stage of E11, or by the tissues analyzed: total RNAs isolated from total head region were used in previous study, whereas only the palatal tissues were used here.

These results altogether imply that exposure to RA during palate development causes facial deformity including cleft palate and cleft lip by modulating the expression of *Hoxa7* as well as an apoptosis-related gene, *Bax*, and thus malregulating the apoptosis during palatogenesis.

Acknowledgements

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