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Original

The Study of Palatal Cell Proliferation and Apoptosis in Retinoic Acid Induced Mouse Cleft Palate Varied with Different Developmental Stage

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Abstract:To determine the mechanisms and patterns of cleft palate induced by varied retinoic acid (RA) in different developmental stages of secondary palate, we detected the cell proliferation and apoptosis of palatal shelves by BrdU and TUNEL labeling on gestation day (GD) 10 and GD12 which RA was exposed, respectively. In GD10 RA treated group, the percentage of BrdU positive cells of embryonic palatal mesenchyme were lower than control group, while TUNEL expression was higher than control group. No significant difference was detected in GD12 RA treated group and in control group either in embryonic palatal mesenchyme or medial edge epithelium by BrdU and TUNEL labeling. Our data indicated that the mechanisms of RA induced cleft palate in the early developmental stage were the inhibition of proliferation of palatal mesenchymal cells and medial edge epithelium cells, as well as excessive induction of apoptosis.

Key words: Retinoic acid, Cleft palat, animal model, Cell proliferation, Apoptosis

Introduction

Cleft lip and/or cleft palate (CL/P) is one of the most common malformations among live births (65% of all malformations). The incidence of cleft palate in China is 1.82% [1]. Its exact mechanism is still unclear because it is considered a kind of multigenetic disease, where a lot of genes and environmental factors are involved. There are three important stages in the development of bilateral palate shelves: rapid horizontal growth, elevation of the tongue, and fusion [2, 3]. In early developmental stage, contact failure of the palatal shelves is due to the inadequacy of palatal elevation or delayed development. Mainly, this is related to the proliferation of embryonic palate mesenchymal (EPM) cells, which constitute the main part of palatal shelves and take part in the formation of bone, muscle and vessel [1][4-6] . In late developmental stage, even though the palate shelves could contact with each other, could not fuse or split after fusion. This is the result of abnormal transformation of the medial edge epithelium (MEE) cells of palate.

The gene homology of human and mice is more than 99%, thus using mouse models as cleft palate model to study the mechanism of cleft palate is recently a trend. Retinoic acid (RA), an endogenous metabolite of vitamin A, plays an important role in embryogenesis by regulating morphogenesis, cell proliferation and differentiation, and extracellular matrix production [7-10]. When the pregnant mouse is exposed to a certain volume of RA, the cleft palate occurs on their embryos. Some researchers believe that extra RA restrains EPM cells proliferation and MEE cells transformation inducing cleft palate. However, the mechanisms and patterns of cleft palate induced by RA in different developmental stages of palate are still unclear.

The objective of this research was to establish stable and high incident cleft palate (CP) mouse model induced by all-trans retinoic acid (RA) on GD10 and GD12, and to explore the mechanism of cleft palate induced by RA on different stages using BrdU for cell proliferation and TUNEL for apoptosis in EPM and MEE cells.

Materials and Methods

Animals and All-trans Retinoic Acid Treatment

C57BL/6J mice were used in this study. The pregnant animals dosed on the noon of gestation day (GD) 10 and GD 12 (day in

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which was found the cervical plug was designated GD 0) with all-trans retinoic acid (RA) (Sigma–Aldrich, St. Louis, MO) 100 mg/kg body weight, respectively. RA was suspended in 0.8 ml corn oil and mixed vigorously to obtain a uniform suspension. Mice were dosed by gavages with 0.2 ml of the suspension. Control animals were dosed with equivalent volume of the carrier alone. Totally 32 pregnant females were obtained and randomly divided into three groups: GD 10 day RA-treated group (n=16) and GD 12 day RA-treated group (n=8), as well as control group (n=8). Embryos were harvested at embryonic 15 days (E15), dissected with PBS, fixed with 4% paraformaldehyde/ PBS at 4 °C, embedded with paraffin, and 4 μm thick in series frontal and cross sections were cut for histological examination, cell proliferation and apoptosis detections.

Cell Proliferation Assay

Pregnant mice were injected intraperitoneally with BrdU (100 mg/kg, Sigma–Aldrich, St. Louis, MO) and sacrificed after 20 minutes of injection. Cell proliferation was detected by BrdU labeling using BrdU staining kit (Zymed, San Francisco, CA). The sections were incubated with trypsin, denaturing solution, blocking solution and incubated with biotinylated mouse anti-BrdU monoclonal antibody. Diaminobenzidine/H_2O_2 (DAB) was used as chromogen for developing the color and counterstaining was performed with hematoxylin.

Apoptosis Detection

TUNEL assay was carried out to detect cell apoptosis with a TUNEL system kit (Takara, Dalian, China) according to the manufacturer's instruction. The sections were pre-treated with Proteinase K(10μg/ml), incubated with the TUNEL reaction mixture with TdT Enzyme and then Anti-FITC HRP conjugated at 37°C. DAB was also used as the chromogen.

Statistics Analysis

The total EPM cells in a determined area near the tip of the palatal shelves (120μm× 120μm) were counted and the ratio of BrdU- positive cells to the total cells was calculated per section in E15 as BrdU index. Each group of BrdU labeling rate was compared using chi-square test by application of SPSS14.0 statistical software.

Results

1. Patterns of cleft palate in different developmental stages induced by RA treatment

The morphology of all embryos and their palates were observed by microscope. In control group, 40 embryos were obtained with normal bilateral palatal shelves fuse, limbs and tails. After exposure of embryonic mice to RA on GD 10, 75 embryos were obtained, all of them showed abnormal palatal shelves formation with small size and the palatal shelves failed to elevate and fuse; 3 embryos show truncated limbs and 2 have shortened tails. After exposure of embryonic mice to RA on GD 12, 40 embryos were obtained, no malformation were detected in limbs and tails. All of the palate shelves were elevated, but showing either failure to contact and fuse or contact but no fusion, which was observed as sub- fissure.

In palatal fusion stage of E15, histological changes were observed by light microscope. Control group showed horizontally growing bilateral shelves contact. Medial edge epithelium (MEE) cells disrupted into epithelial islands, part of them disappear, and bilateral mesenchyme go through each other. RA induced cleft palate in GD 10 group (10RA group) showing vertically growing bilateral shelves beside the tongue, and failed to contact. MEE cells still maintained bilayer. RA induced cleft palate in GD 12 group (12RA group) showing horizontally growing bilateral shelves contact. MEE cells grow into epithelium cord, the epithelium cord seldom disrupt or disappear and less bilateral mesenchyme go through each other.

2. Study of cell proliferation by BrdU expression in embryonic day 15 during palatal fusion stage

Proliferation cells were detected by positive reaction to BrdU within the nuclei. In control group, BrdU positive cells were located in both palatal mesenchyme and epithelium diffusely (Fig.1 A, B& C). In 10RA group, distribution pattern was similar to the control group but less BrdU positive cells were detected (Fig.1 D, E& F). In 12RA group, there was no significant difference in the expression level and distribution of BrdU positive cells compared with control group (Fig.1 G, H& I).

The percentage of BrdU positive cells of 10RA embryonic palatal mesenchyme (EPM) was lower than that of control group in both frontal and cross sections ($P<0.01$). There was no difference between GD12 group and control group ($P>0.05$) (Fig.2).

3. Study of apoptosis by TUNEL expression in embryonic day 15 during palatal fusion stage

Apoptotic cells were also detected by TUNEL, showing specific positive localization in the nuclear. in control group, TUNEL positive cells specifically located in MEE cells, but not detected in palatal mesenchyme cells (Fig.3 A& B). In 10RA group, increase of TUNEL positive cells distributed in palatal mesenchyme was strongly expressed, but less TUNEL positive cells in medial edge epithelium was detected (Fig.3 C& D). In 12RA group, there was no significant difference in the expression level and distribution of TUNEL positive cells compared with control group (Fig.3 E& F).

Discussion

Retinoic acid (RA), the derivative of vitamin A, plays an important role in regulating embryogenesis, morphogenesis, cell proliferation and differentiation, maintaining normal physiological

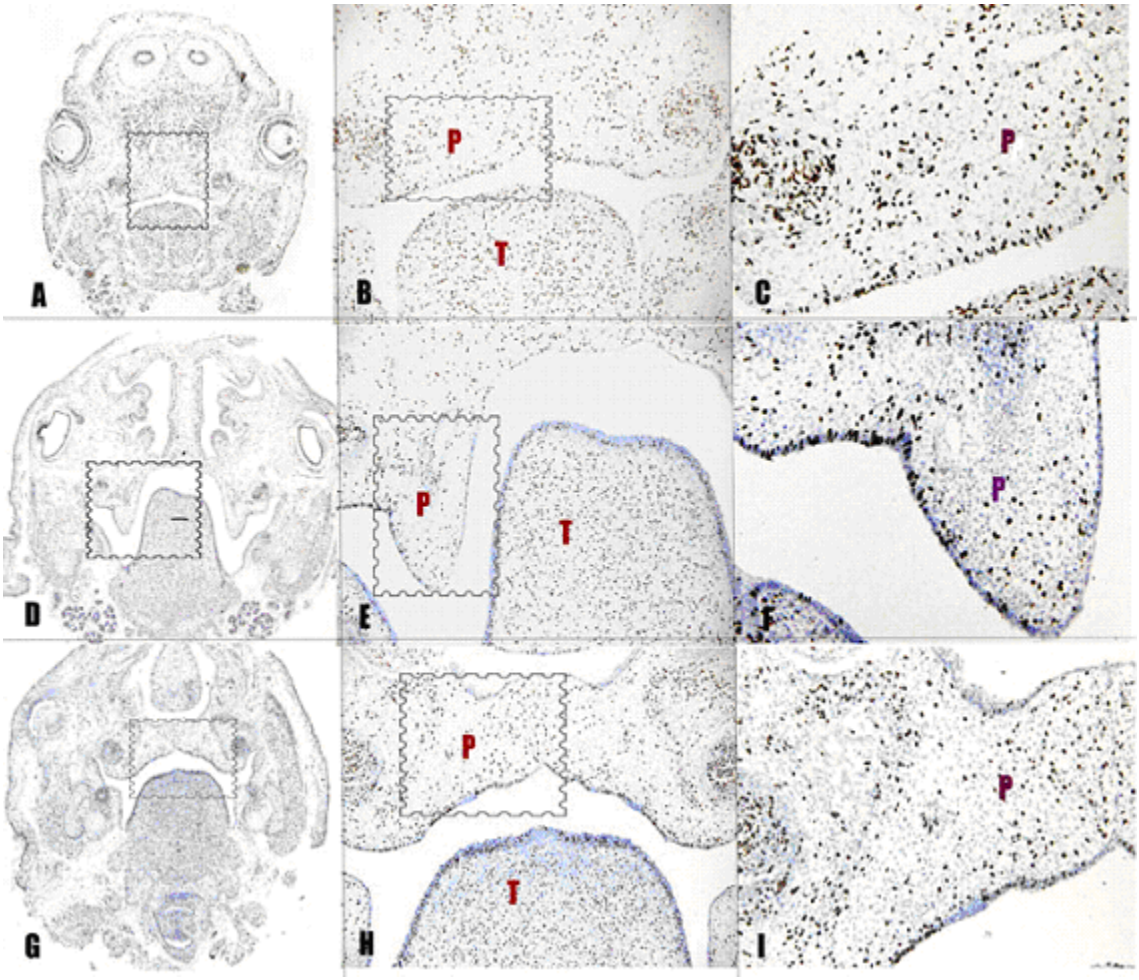

Figure 1. BrdU expression for cell proliferation in frontal section of palate fusion stage. In control group, BrdU positive cells locate in both palatal mesenchyme and epithelium diffusely (A, B and C). In 10RA group, distribution pattern is similar to the control group but less BrdU positive cells are detected (D, E and F). In 12RA group, there is no significant difference in the expression level and distribution of BrdU positive cells compared with control group (G, H and I).

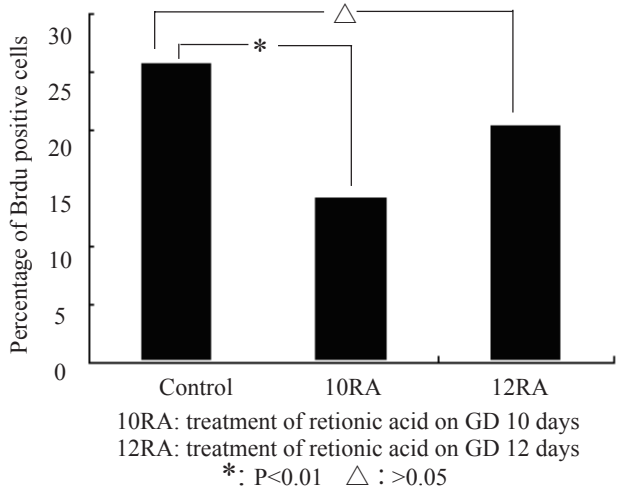

Figure 2. Percentage of BrdU positive cells in embryonic palatal mesenchyme in the fusion stage. The percentage of BrdU positive cells of 10RA embryonic palatal mesenchyme (EPM) is obviously lower than the control group in both frontal and cross sections (P<0.01). There is no difference between GD12 group and control group (P>0.05).

steady state. Ligand of RA combines with its receptors (RXRα and RARβ) and RA binding proteins (CRABPs), activates its transcription factor-AP2, then increases c-jun and c-for gene expression to block cell cycle at the G0/G1 phase, and induces the mature differentiation of cells. It has been proved that RA has high teratogenicity in embryogenesis and may induce a variety of abnormalities, including neural tube defects, craniofacial abnormalities, and limb defects [11, 12]. AP2 gene specifically is expressed in mouse maxillofacial region during its developmental stages, and cleft lip/ palate occurred when the mouse AP2 gene was knocked out. RA also has close relationship with the signal pathways during the process of cleft palate formation. RA may increase the expression of EGF receptor in palatal shelves, promotes EGF binding with its receptor to prevent MEE apoptosis and promote oral epithelial-like differentiation. Some researchers also mentioned RA induced some malformations through the BMP signaling pathway to induce excessive apoptosis [13].

Development of palate is a complex process, which is a strict

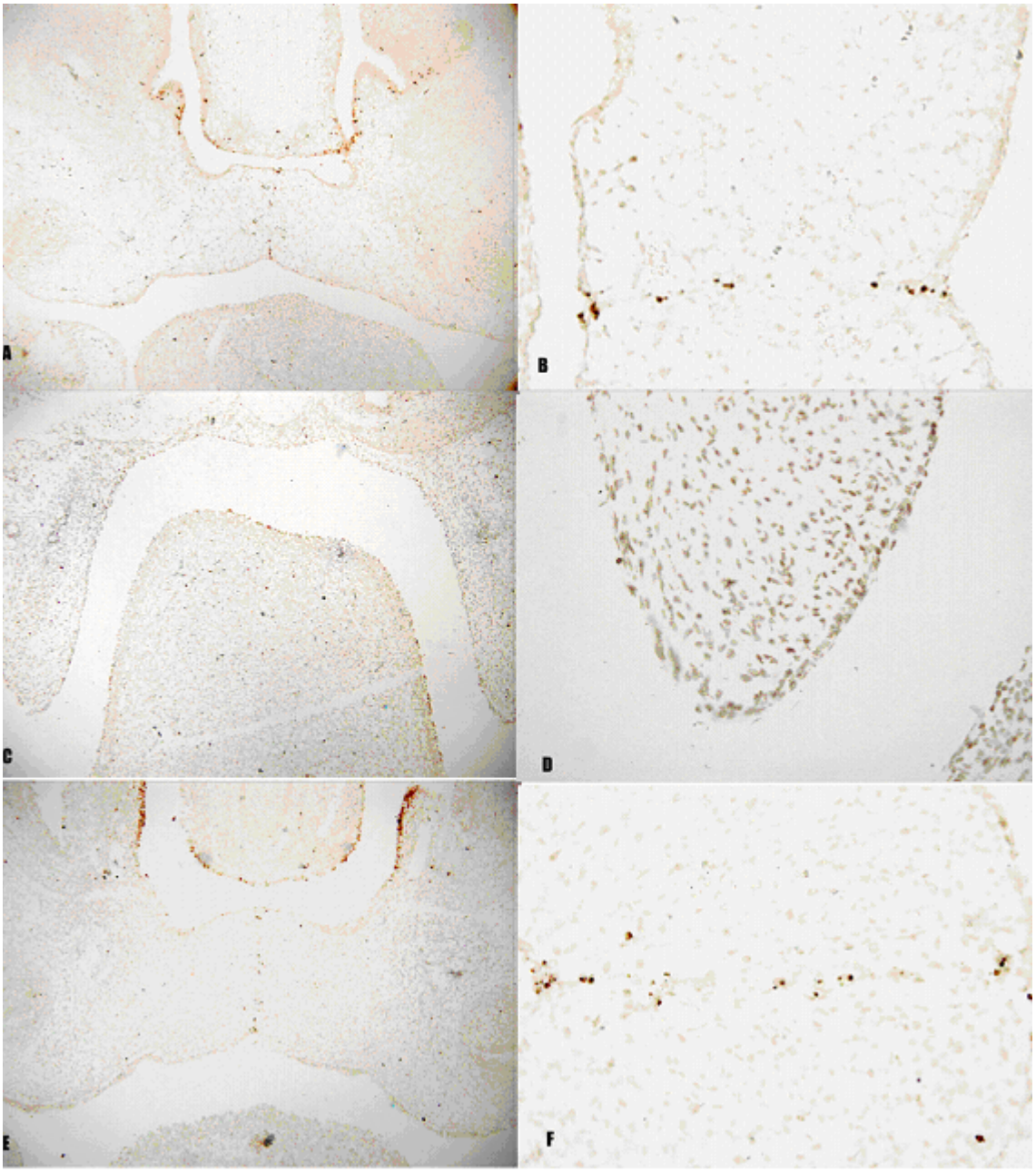

Figure 3. TUNEL expression level of apoptosis cells in frontal section of palate fusion stage. In control group, TUNEL positive cells are specifically located in MEE cells, but not detected in palatal mesenchyme cells (A and B). In 10RA group, increased the expression of TUNEL positive cells distribute in palatal mesenchyme, but less TUNEL positive cells in medial edge epithelium are detected (C and D). In 12RA group, there is no significant difference in the expression level and distribution of TUNEL positive cells compared with control group (E and F).

discipline and performs a precise temporal and spatial sequence, including cell proliferation and differentiation, and mutual recognition. In normal EPM cells, there will be a small amount of apoptotic cells which are scattered distributed and are able to remove individual EPM cells in lesion. RA produces a mass of apoptotic cells in EPM during an exuberant cellular proliferation stage, impacting the normal development of the palate in shape and size, which is one of the reasons for cleft palate formation. In our experiment, in GD 10 day RA-treated group (the pre-developing stage of palate), abundant exogenous RA lead an inhibition of EPM cell proliferation and abnormal apoptosis of EPM cells in early stage of palate development, truncation of bilateral palatal shelves and failure to contact at the late stage of palate fusion. While in GD 12 day RA-treated group (rapidly growing stage of palate), intaking of the same amount of RA compared with GD 10 RA-treated group, there are no excessive apoptosis in EPM cells at fusion stage, and no significant difference in the shape and size of palate compared with control group

showing bilateral palatal shelves get rise and contact. This suggests that RA perform different influence to the apoptosis of EPM cells at different developing stages. RA acting on the pre-developing stage induces excessive apoptosis of EPM cells and finally leads to a truncated palatal shelves and cleft palate.

Currently, there are three ultimate outcome forms of MEE cells: apoptosis, epithelial-mesenchymal transformation and migration to the oral or nasal epithelium. Mori et al. demonstrated that apoptotic programmed cell death occurs at some stages of palatal fusion, although the present results do not exclude the possibility of epithelial-mesenchymal transformation and the oral and nasal migration of midline epithelial cells [14]. Other researchers thought that apoptosis occurred with epithelial-mesenchymal transformation. Their data showed that MEE cells underwent apoptosis during palatal formation, even though they migrated into epithelial triangles or transformed into mesenchymal cells. Moreover, apoptotic bodies and cellular debris were phagocytosed by adjacent MEE cells or mesenchymal cells and digested by lysosomal enzymes [15]. Some researches proposed the epithelial-mesenchymal transformation theory and demonstrated that MEE cells died and transformed into mesenchyme during palatal fusion and that dead cells were phagocytosed by macrophages [16]. Shuler et al. marked MEE cells by means of Dil, indicated that MEE did not die but underwent a phenotypic transformation to viable mesenchymal cell types [17]. The expression of á smooth muscle actin in MEE cells during bone formation stage also proved the epithelial-mesenchymal transformation [18]. Carette et al. used cytokeratin labeling as marker for palatal epithelial differentiation showing that cytokeratin 19 was absent specifically from the medial edge epithelial cells. This may be related to the loss of cytokeratin expression observed during epithelial-mesenchymal transformations [19].

In our experiment, bilateral palate shelves elevate and contact in control group and in 12GD RA-treated group, TUNEL cells scatter in MEE cells. In contrast, palate shelves were truncated and could not contact in 10GD RA-treated group, and no TUNEL cells were detected in MEE cells. No apoptosis was detected in MEE cells when the bilateral palatal shelves failed to contact and fuse in GD10 RA-treated group. This suggests the apoptosis occur after contacting of the palatal shelves. Our data also indicated that there was no significant difference in the positive rate of TUNEL and BrdU in GD12 RA-treated group compared to the control group, and the palatal medial ridge epithelium was relatively intact. This study suggests that treating pregnant mice with RA on GD 12 may affect the transformation of MEE cells, without the induction of apoptosis.

In summary, exposition of RA induced different pattern of cleft palate varies during the developmental stages. After exposure of embryonic mice to RA on GD 10, the proliferation and differentiation of palatal mesenchymal cells were inhibited, which contributed to small size of shelves and failure to contact. The MEE cells maintained a bilayer midline epithelial seam with normal apoptosis in GD 12, which could indicate that cell apoptosis in MEE cells is not the only process required for palatal fusion.

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