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All-*trans* Retinoic Acid Promotes Cell Apoptosis Through Gamma Amino Butyric Acid Pathway in Murine Embryonic Palate Mesenchymal Cells

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Abstract Retinoic acid (RA) is a teratogen which can induce cleft palate. Recent studies suggested that gamma amino butyric acid (GABA) was involved in the development of palate. If the GABA signal pathway participates the cleft palate induced by RA remains to be elucidated. In the present study, we investigated the effect of all-*trans* retinoic acid (atRA) (0.2, 0.67, 2.0 and 6.7 μmol/L) on cell proliferation and apoptosis, and then examined the role of gamma amino butyric acid (GABA) signaling pathway in regulation of cell proliferation and apoptosis by atRA in murine embryonic palate mesenchymal (MEPM) cells. Results showed that atRA (2 μmol/L and 6.7 μmol/L) significantly inhibited cell proliferation and increased apoptosis. The mRNA and protein expression of glutamic acid decarboxylase 67 (GAD 67) which was a key enzyme in synthesis of GABA were significantly down regulated by atRA (0.67, 2.0 μmol/L and 6.7 μmol/L). But the mRNA and protein expression of GABAAR-β3 were shown no obvious change compared with the control group. When GABA (1.0 μmol/L) was added to cell culture system, the effect of atRA (6.7 μmol/L) on the proliferation and apoptosis of MEPM cells was reversed. In conclusion, all-*trans* retinoic acid inhibits cell proliferation and promotes cell apoptosis through gamma amino butyric acid pathway in murine embryonic palate mesenchymal cells.

Key words all-*trans* retinoic acid, murine embryonic palate mesenchymal cell, glutamic acid decarboxylase, gamma amino butyric acid, proliferation, apoptosis
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Glutamic acid decarboxylase (GAD) catalyzes synthesis of the inhibitory neurotransmitter gamma amino butyric acid (GABA) in brain. The GABA-ergic neurons of the mammalian nervous system express two homologous forms of GAD, with protein sizes of 67 and 65 ku, respectively, each encoded by a different gene [1]. Several different properties of these isoforms have been suggested that they have distinct roles in neural functions [2]. The knock-out of each GAD isoform by gene-targeting is expected to settle these issues. The recent generation of GAD 65 -/- mice yielded the unexpected results that lack of GAD 65 does not change brain GABA contents[3-4]. It is estimated that GAD 67 accounts for 56%～85% of the GABA synthesis flux in rat cerebral cortex at baseline [5], and up to 80%～90% of overall GABA levels in mouse brain[6-7]. Studies in genetically engineered mice revealed that the two GAD isoforms played different roles during development. Loss of GAD 67 (Gad1) was not compatible with postnatal life due to severe defects in craniofacial development. GAD 67 null mutant mice succumbed shortly after birth due to complications from cleft palate in conjunction with malfunction of the central respiratory control system[6,8-9]. In contrast, genetic ablation of GAD 65 (Gad2) in mice did not result in major craniofacial malformations but increased the brain's susceptibility to seizures[3-4].

Gamma amino butyric acid type A receptors (GABAARs) are pentameric chloride channels

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assembled from 19 different subunits, α1～6, β1～3, γ1～3, δ, ε, π, ρ1～3, and θ [10–11]. Most native receptors are formed from 2α, 2β, and 1γ or 1δ subunits [12]. The β3 subunit is especially interesting because this subunit has been suggested to be a candidate gene for neurodevelopmental disorders such as Angelman Syndrome and autism spectrum disorder[13–15]. GABAAR-β3 is also a crucial site where intravenous anesthetics and ethanol present their action [16–18], and is an important receptor involved in developmental processes [19]. It has been demonstrated that global β3- knockouts mice appeared substantial alteration of incidence of cleft palate[20]. The mice line with a conditionally inactivated β3 gene also showed increased incidence of cleft palate [21]. So the GABA type A receptors must take part in the development of palate.

Retinoic acid (RA) is derived from vitamin A1 or retinol, and is a morphogen and is involved in vertebrate limb and organ development[22]. All-*trans*-RA initiates signaling by binding to the retinoic acid receptor (RAR) in association with the auxiliary 9-*cis*-retinoic acid receptor (RXR). The liganded RAR/RXR complex recognizes specific promoter response elements (RARE) to regulate transcription of target genes. The transcriptional regulatory role of the transcription factor AP-2 is intimately linked to retinoic acid-induced differentiation since AP-2 is induced by retinoids [23]. The retinoids can trigger cell differentiation and block malignant progress, perhaps because they also negatively regulate POU-domain factors such as Oct-3 [24] and AP-1-responsive genes [25]. Retinoids induces the differentiation of embryonal carcinoma cells into neurons [26] and regulates the expression of GABA receptors and glutamate decarboxylase in different cells [27–28]. It is clear that retinoic acid can induce cleft palate through different cell signal transduction pathways, such as TGF and PDGF [29–31]. Based on the information provided above, GABA-ergic pathway has interacted with retinoic acid in organism and it may also take part in the biological effect of retinoic acid. So we carried out this work to find more information about the cleft palate induced by retinoic acid and to elucidate whether GABA-ergic pathway is involved in the malformation of palate induced by retinoic acid.

1 Materials and methods

1.1 Cell culture

Murine embryonic palate mesenchymal (MEPM) cells were derived from palatal tissue of gestational day 11 ICR mouse embryos (date of vaginal plug detection was judged as day 0 of gestation). The palate shelves were dissected in sterile and cold phosphate-buffered saline (PBS). Then they were immersed in PBS (contained 0.25% trypsin/0.05% EDTA) and minced in stainless steel mesh which had 100 holes in each square centimeter. The suspension was incubated at 37℃ for 2 min with constant shaking. When digestion was finished, the suspension was filtered with stainless steel mesh which had 400 holes in each square centimeter. Fetal bovine serum (FBS) was added into filtered suspension in order to devitalize the trypsin. Cells were collected by centrifuge and washed with PBS twice. At last cells were adjusted to certain concentration for different purposes.

1.2 Cell treatment

MEPM cells were resuspended in DMEM culture medium (Gibco) supplemented with 10% FBS (Hyclone) at a density of 1.0×10^6 cells/ml. One hundred microliter of cell suspensions was added to each well of 96 wells cell culture plate. After 24 h incubation at 37℃ with 5% CO_2, supernatant was removed and all-*trans* retinoic acid (atRA) (Sigma) was added to each well at different concentration (the final concentrations of atRA were 0.2, 0.67, 2.0 and 6.7 μmol/L). Cells were cultured with atRA for 24 h, and the viability was tested. For cell apoptosis and Western blot analysis, MEPM cells were resuspended in DMEM culture medium supplemented with 10% FBS at a density of 5.0×10^5 cells/ml. Then 1.5 ml (for cell apoptosis) and 3 ml (for Western blot analysis) cell suspension were added to each culture dish. After 24 h culture at 37℃ 5% CO_2 condition the supernatant was removed and the culture medium which contained atRA in different concentrations was added in each dish. Then cells were cultured with atRA for 24 h.

In order to observe the effect of GABA supplementation on the proliferation and apoptosis of MEPM cells induced by atRA, GABA (Sigma) was added to culture system with atRA at the same time.

The concentration of GABA was selected through preliminary test and it was 1.0 μmol/L.

1.3 Viability of cells

The viability of cells was measured with the MTT assay. This assay is based on the ability of living and metabolically active cells to convert the yellow tetrazolium salt(MTT[3-(4, 5-dimethyldiazol-2-yl)-2, 5 diphenyl tetrazolium bromid]; Sigma) into a blue formazan product. The cells were co-cultured with MTT (0.5 g/L) for 4 h at 37℃ and then the dark blue crystals was dissolved using lysis buffer (4 ml 1 mol/L HCl and 96 ml isopropyl alcohol) for 30 min. The optical density was measured by a microplate reader (Dynatech, Denkendorf, Germany) at 570 nm.

1.4 Cell apoptosis

All treated cells were transferred to EP tube after treatment. Cells were washed with pre-cold PBS two times. Cell suspension was centrifuged under 1 800 r/min for 5 min in order to separate the cells and supernatant. The density of cells was adjusted to 1×10^5/ml. 25 μl cell suspension was stained with 2 μl acridine orange (AO) / ethidium bromide (EB) mixture (100 μg/L AO and 100 μg/L EB). Then the solution was put on the slide and observed under the fluorescence microscope. The apoptotic cell percentage was calculated.

The cell apoptosis was also detected by the flow cytometry. When exposure was finished, cells were washed with PBS twice. 500 μl binding buffer was added and then 10 μl FITC-labeled Annexin-V (20 mg/L) was added. The cells were kept in darkness for 30 min at room temperature. Then 5 μl propidium iodide (PI, 50 mg/L) was added and incubated for 5 min. Then cells were resuspended with 1 × binding buffer at a concentration of 10^6 cells/ml. Finally they were incubated with 10 μl fluorescein-conjugated Annexin V and 5 μl PI. Annexin V binds to cells expressing phosphatidylserine on the outer layer of the cell membrane whereas PI stains the cellular DNA of cells with a compromised cell membrane. This allowed live cells (unstained with either fluorochrome) to be discriminated from earlier apoptotic cells (stained only with Annexin V) and later apoptotic cells (stained with both Annexin and PI). Cells were detected by flow cytometry after cells were digested with trypsin and washed twice in PBS. The apoptotic cell percentage was calculated.

1.5 RNA isolation and real-time quantitative PCR analysis

Total RNA from cells was extracted by using Trizol reagent according to the manufacturer's instructions. Real-time quantitative PCR, using SYBR Green detection chemistry, was performed on Roche light Cycler Run 5.32 Real-Time PCR System. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the $\Delta\Delta Ct$ method and expression of GAPDH was used as the internal control. The mRNA expression of the cotrol group was expressed as 100% . Fold-induction of mRNA expression was calculated. The oligonucleotide primers were used for murine GAD 67: forward primer, 5′ CAT GGC TGC TCG TTA CAA GTA 3′, reverse primer, 5′ AAC AGT CGT GCC TGC GGT TGC 3′. GABAAR-β3 forward primer: 5′GAC CGT TCA AAG AGC GAA AG 3′, reverse primer: 5′ CGT AGA TGG GTC TTC TTG TGC 3′; GAPDH forward primer: 5′ TTC CTA CCC CCA ATG TAT CCG 3′, reverse primer: 5′ ACC ACC CTG TTG CTG TAG CCA 3′.

1.6 Western blot analysis

MEPM cells were lysed in protein extracting buffer (0.5% NP-40, 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, and 50 mmol/L NaPPi) in the presence of a protease inhibitor (1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 150 nmol/L aprotonin, 1 μmol/L leupeptin, 1 μmol/L E-64 protease inhibitor and 500 μmol/L 4- (2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride(AEBSF)). Protein concentration was measured using Bradford analysis. Equal amounts of protein were separated by 6%～10% SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were blocked with Tris buffered saline with tween (TBST) (20 mmol/L Tris/ glycine, pH 7.6, 137 mmol/L NaCl, 0.1% Tween 20) and 5% dried milk. Membranes were probed with primary antibody(GAD67 and GABAAR-β3) (SANTA CRUZ) diluted to the suitable concentration in TBST/5% dried milk at 4℃ overnight. After extensive

washing with TBST, the membranes were probed with a horseradish peroxidase-linked secondary antibody (SANTA CRUZ) diluted 1 ∶200 in TBST/5% dried milk at room temperature for 2 h. At last after washing with TBST, the signal was visualized by chemiluminescence (ECL) (Amersham Pharmacia Biotech; Piscataway, NJ).

1.7 Statistical analysis

All data were presented as $\bar{x} \pm s$. The data were evaluated by one-way ANOVA followed by least significant difference (LSD) test as a post hoc test. Statistical significance was at $P < 0.05$.

2 Results

2.1 Effect of all-trans retinoic acid (atRA) on the cell proliferation of murine embryonic palate mesenchymal (MEPM) cells

After treatment with all-*trans* retinoic acid (0.2, 0.67, 2.0 and 6.7 μmol/L) for 24 h, all-*trans* retinoic acid (2.0 and 6.7 μmol/L) significantly inhibited the proliferation of MEPM cells compared with the control group. As the concentration of all-*trans* retinoic acid increase the viability of MPEM cells were decreased (Figure 1a).

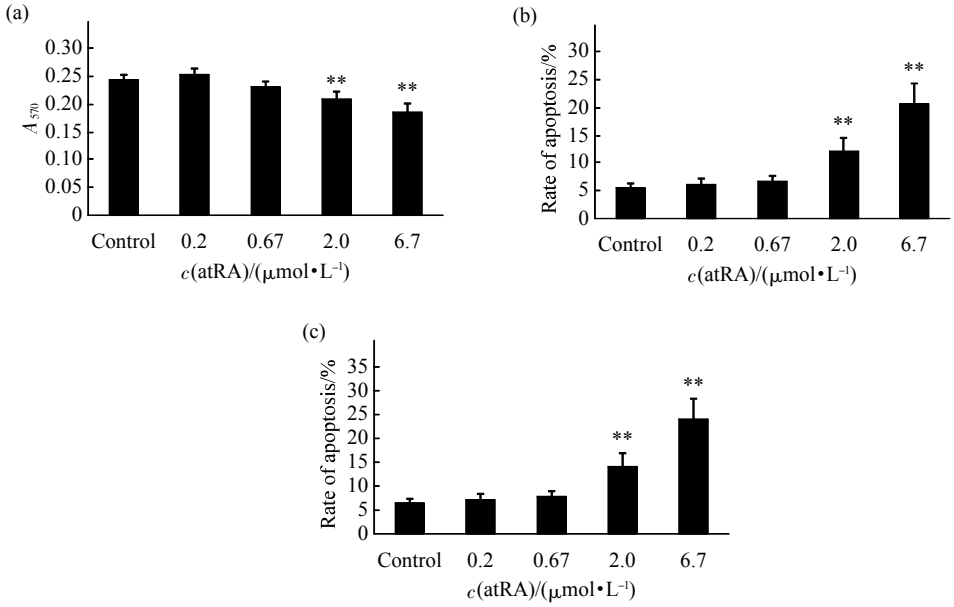

Fig. 1　Effect of all-*trans* retinoic acid (atRA) on the cell proliferation and apoptosis of murine embryonic palate mesenchymal (MEPM) cells

(a) Viability of MPEM cells was detected by MTT assay. (b)The cell apoptosis was detected by the fluorescence microscope. (c) The cell apoptosis was detected by the flow cytometry. Control refers to cells treated with 0 mol/L atRA. Data are expressed as $\bar{x} \pm s$ ($n = 6$). $^{**}P < 0.01$, *vs* the control group.

2.2 Effect of atRA on the apoptosis of MEPM cells

After treatment with all-*trans* retinoic acid (0.2, 0.67, 2.0 and 6.7 μmol/L) for 24 h, the rate of MEPM cells apoptosis was examined by AO/EB staining and PI staining. Compared with the control group, the apoptosis rate was significantly increased in 2.0 and 6.7 μmol/L atRA group (Figure 1b, c).

2.3 Effect of atRA on the GAD 67 and GABAAR-β3 mRNA level in MEPM cells

After treatment with atRA (0.2, 0.67, 2.0 and 6.7 μmol/L) for 24 h, the mRNA level of GAD 67 was tested by real-time PCR. The mRNA level of GAD 67 was decreased gradually with atRA concentration increasing. Compared with the control group, the mRNA level of GAD 67 was significantly decreased in 0.67, 2.0 and 6.7 μmol/L atRA group (Figure 2a). But the mRNA level of GABAAR-β3 was not influenced by atRA (0.2, 0.67, 2.0 and 6.7 μmol/L) (Figure 2b).

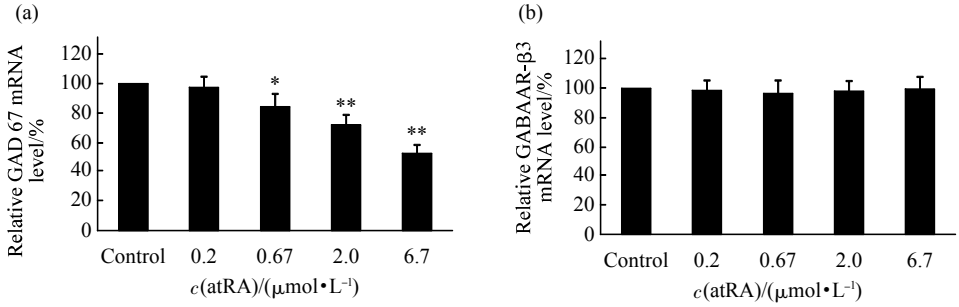

Fig. 2 Effect of all-*trans* retinoic acid (atRA) on the mRNA level of GAD 67 and GABAAR-β3 in MPEM cells

GAD 67 and GABAAR-β3 mRNA expression were measured by real-time quantitative PCR. The mRNA expression of the cotrol group was expressed as 100%. Fold-induction of mRNA expression was calculated. Control refers to cells treated with 0 mol/L atRA. Data are expressed as $\bar{x} \pm s$ ($n = 6$). *$P < 0.05$, *vs* the control group; **$P < 0.01$, *vs* the control group.

2.4 Effect of atRA on the GAD 67 and GABAAR-β3 protein in MEPM cells

The protein GAD 67 level was reduced gradually with the increased atRA concentration. Compared with the control group, the protein level of GAD 67 were lower in 0.67, 2.0 and 6.7 μmol/L atRA group (Figure 3a, c). At the same time the protein level of GABAAR-β3 was not influenced by atRA(0.2, 0.67, 2.0 and 6.7 μmol/L) (Figure 3b, d).

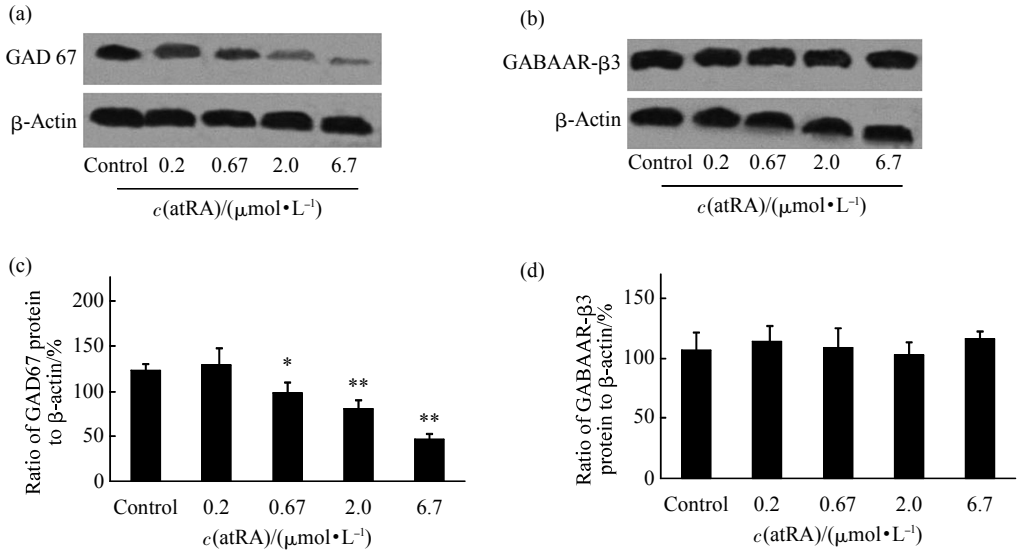

Fig. 3 Effect of all-*trans* retinoic acid (atRA) on the protein level of GAD 67 and GABAAR-β3 in MPEM cells

(a, b) The representative image of the changes in protein expression levels of GAD67 and GABAAR-β3 shown by Western blot in MPEM cells. (c, d) Histogram shows the levels of GAD 67 and GABAAR-β3 protein expression in MPEM cells determined by densitometric analysis. Control refers to cells treated with 0 mol/L atRA. Data are expressed as $\bar{x} \pm s$ ($n = 6$). *$P < 0.05$, *vs* the control group; **$P < 0.01$, *vs* the control group.

2.5 Effect of GABA on changes of cell proliferation and cell apoptosis induced by atRA

As shown in Figure 4a, atRA (6.7 μmol/L) significantly decreased the proliferation of MEPM cells compared with the control group. However, GABA (1.0 μmol/L) reversed the effect of atRA (6.7 μmol/L) on the proliferation of MEPM cells. Similar result was observed about cell apoptosis of

MEPM cells which GABA (1.0 μmol/L) reversed the incremental effect of atRA (6.7 μmol/L) on the apoptosis of MEPM cells (Figure 4b, c).

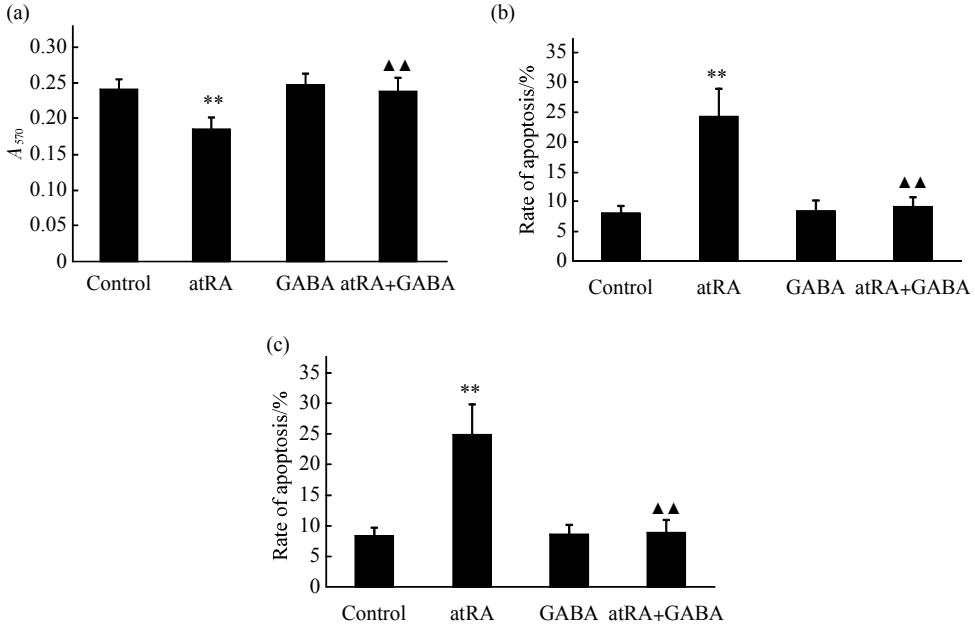

Fig. 4 Effect of atRA (6.7 μmol/L) and GABA (1.0 μmol/L) on the proliferation and apoptosis of MPEM cells

(a) Cell viability was detected by MTT assay. (b) The cell apoptosis was detected by the fluorescence microscope. (c) The cell apoptosis was detected by the flow cytometry. Control refers to cells treated with 0 mol/L atRA and GABA. Data are expressed as $\bar{x} \pm s$ (n=6). **$P < 0.05$, *vs* the control group; ▲▲$P < 0.05$, *vs* the atRA (6.7 μmol/L) group.

3 Discussion

Development of palate is the process of growth and fusion of prominences and involves cell migration, proliferation, differentiation and apoptosis. The mesenchymal core derives from the cranial neural crest and has an epithelial outer covering derived from ectoderm. During the process of palate development, the main changes are cell proliferation and cell migration. And eventually cell conglutination is also important for palate formation. So if the environmental factors influence any ring-joint of palate development mentioned above, there may appear malformation of palate and eventually induce cleft palate. It is well known that, during the organogenesis of embryo, the vitamin A overload of mother may induce different kinds of malformation, such as cleft palate. Many researches have identified that the main component caused the adverse effect of vitamin A is retinoic acid. Some researches observed the correlation between cleft palate and γ-amino butyric acid (GABA) pathway in mice treated with retinoic acid (RA)[32–33]. However, if GABA pathway is involved in cleft palate induced by RA is not clear. In order to clarify this hypothesis, we obseved the effect of all-*trans* retinoic acid (atRA) on murine embryonic palate mesenchymal (MEPM) cells proliferation and apoptosis. The result indicated that atRA (2.0 and 6.7 μmol/L) significantly reduced the proliferation of MEPM cells. At the same time the apoptosis of MEPM cells was also increased under these two concentrations. If there is interference of cell proliferation or programed cell death during the palate development, cleft palate will show up [34–35]. So the formation of cleft palate induced by RA might be related with decreased proliferation and incremental apoptosis of cells which were associated with palate development caused by RA.

It is widely accepted that the neurotransmitter γ-aminobutyric acid (GABA) regulates not only neuronal activities but also cell migration, survival, proliferation and differentiation in both neuronal and nonneuronal cells [36]. Glutamic acid decarboxylase 67 (GAD 67) is a key enzyme in synthesis of GABA. Presence of endogenous GABA or GAD 67 has also been demonstrated in the palate shelves (PS)[6, 20]. This hinted that GABA pathway might be involved in the

organogeny of palate. Under the condition that cells proliferation and apoptosis were influenced by atRA, we observed the transcription and translation level of GAD 67. The result indicated that not only the mRNA level but also the protein level of GAD 67 was decreased clearly by atRA. Even at the concentration which is lower to decrease the proliferation and to increase apoptosis of MEPM cells, atRA could still decrease the GAD 67 mRNA and its protein level. It suggested that the GAD 67 may be play the key roles in the progress of MEPM cells proliferation and apoptosis induced by atRA. So we guessed that GABA pathway might be involved in the decreased proliferation and incremental apoptosis of MEPM cells caused by atRA.

The implication of GABA in palate development was demonstrated by genetic studies in mice lacking the β3 subunit of GABAA receptor (GABAAR-β3). Mice lacking GABAAR-β3 developed cleft palate without other craniofacial malformations[6-7, 37]. Generally the process of palate development is divided into three stages. The first stage is palatal shelf growth. The second is palatal shelf elevation and the third is palatal shelf adhesion/fusion. Hyaluronan forms gels after binding water and elicits tissue expansion of the PS. Hyaluronan also regulates cell proliferation and migration. Proteoglycan-glycosaminoglycans (PG-GAG) is crucial for proliferation and polarized alignment of PS mesenchymal cells. Collagen fibers may also control cell alignment. GABA, synthesized from glutamic acid (Glu) by GAD 67, binds to GABAA receptor β3 subunit. GABA signaling may elicit a range of biological activities, including cell proliferation and migration necessary for PS lifting[29]. Picrotoxin is an antagonist of the GABAA receptor, and 3-mercaptopropionic acid (3-MP) is a comparatively specific inhibitor of GAD. Both of them induced cleft palate at high rate——formation of the secondary palate was obstructed completely or partially [38]. However, our results showed that both mRNA level and the protein level of GABAAβ3 were not influenced by atRA. Thus the decreased proliferation and incremental apoptosis of MEPM cells caused by atRA was not mediated through the change of GABAAR-β3 level.

The GAD 67 was decreased after the atRA treatment and then the GABA content would be lower in MEPM cells. The GABA is the essential signal molecule for cell proliferation and programmed cell death, so the GABA reduction would disturb the proliferation and apoptosis of MEPM cells. Eventually the palatal shelf growth would be broken down. So we tried to add GABA to the cells treated with atRA. After GABA supplementation the inhibited cell proliferation and increased cell apoptosis were relieved. So the inhibitory proliferation and incremental apoptosis of MEPM cells induced by atRA were related to the decrease of GAD 67 expression which led to the decrease of GABA level.

In conclusion, all-*trans* retinoic acid inhibits cells proliferation and promotes cells apoptosis through gamma amino butyric acid pathway in murine embryonic palate mesenchymal. GABA pathway occupies one site in web of mechanism of RA induced cleft palate.

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全反式维甲酸通过γ-氨基丁酸途径促进小鼠胚胎腭板间充质细胞的凋亡

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摘要　维甲酸(RA)是一种能够诱导腭裂发生的致畸物．研究显示γ-氨基丁酸(GABA)在腭板的发育过程中发挥重要作用．而GABA是否参与了RA诱导的腭裂发生还不清楚．本研究以小鼠胚胎腭板间充质细胞(MEPM)为研究对象，观察全反式维甲酸(atRA)(0.2、0.67、2.0和6.7 μmol/L)对MEPM细胞增殖和凋亡的影响，并探讨GABA信号通路在其中的可能作用．结果显示，atRA(2.0 μmol/L和6.7 μmol/L)显著性抑制了MEPM的增殖，并促进了细胞凋亡．atRA(0.67、2.0和6.7 μmol/L)显著性降低了GABA合成的关键酶谷氨酸脱羧酶(GAD67)mRNA和蛋白质的表达，但对γ-氨基丁酸A型受体-β3(GABAAR-β3) mRNA和蛋白质的表达没有影响．1.0 μmol/L的GABA逆转了atRA(6.7 μmol/L)对MEPM细胞增殖和凋亡的影响．以上结果表明，atRA通过下调GAD67的表达，减少GABA的产生，抑制MEPM的增殖和促进MEPM的凋亡，从而可能影响腭板的发育，诱导腭裂形成．

关键词　全反式维甲酸，小鼠胚胎腭板间充质细胞，谷氨酸脱羧酶，γ-氨基丁酸，增殖，凋亡

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