Induction of Cell-Cycle Arrest by all-trans Retinoic Acid in Mouse Embryonic Palatal Mesenchymal (MEPM) Cells

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INTRODUCTION

In the mouse, secondary palate shelves arise from the developing maxillary process on gestation day (GD) 12.5 by initially growing in a vertical position alongside the tongue and then undergoing rapid elevation to the horizontal position above the tongue, which brings opposing shelves into contact at the midline on GD 14.5 (Eldeib and Reddy, 1988; Ferguson, 1988). At this time, the palate shelves are ready to make contact with each other and thereupon to fuse. During this process of palatalogenesis, maintenance of normal proliferation of palatal mesenchymal cells is very important. It is widely believed that one of the primary causes of cleft palate is failure of palatal processes to elevate as a result of growth inhibition. Control of this process at the cellular level is likely to be mediated by specific intracellular signal transduction mechanisms and cell-cycle inhibitory growth factors secreted in response to various teratogenic microenvironments (Dhulipala et al., 2004).

Retinoic acid, especially all-trans retinoic acid (atRA) plays an important role in embryogenesis, by regulating morphogenesis, cell proliferation, differentiation, and extracellular matrix production (Lai et al., 2003; Sasaki et al., 2004; Shibamoto et al., 2004; Wang and Kirsch, 2002). It is also teratogenic at high concentrations. The induction of cleft palate by atRA varies depending on the stage of development exposed. In vivo studies have indicated that, after exposure of embryonic mice to RA on GD 10, abnormally small palatal shelves form. After exposure on GD 12, shelves of normal size form, but fail to fuse, as the medial cells proliferate and the normal growth and differentiation process of palatal mesenchymal cells is inhibited. Because reduced proliferation of palatal mesenchymal cells can contribute to smaller shelf size, the hypothesis that atRA may have adverse effects on this cellular processes was tested in the present study, using primary mouse embryonic palate mesenchymal cell (MEPM) cultures.

Previous studies have indicated that retinoids suppress cell growth by several cellular mechanisms. They inhibit cell proliferation by inducing G1 arrest in many different cell types. The cell cycle in eukaryotes is regulated by cyclin-dependent kinases (cdk). The cyclins, members of the cell cycle regulators, bind to and activate cdk. Activation of cyclin/cdk is required for cell cycle progression and G1/S transition in response to microenvironments. During progression through G1, the amount of D-type cyclin increases, and, in a mitogen-regulated manner, these proteins associate with and activate cdk4 during early- to mid G1 phase, which phosphorylates Rb on specific residues (including Ser780) (Connell-Crowley et al., 1997; Pan et al., 2001; Zarzowska and Mittnacht, 1997). This releases the activity of E2F toward the cyclin E gene promoter, and consequently, the expression of cyclin E activates cdk2, completing the hyperphosphorylation of Rb (Geng et al., 1996; Kitagawa et al., 1996; Lundberg and Weinberg, 1998; Weinberg, 1995) and allowing the G1/S transition (Donnellan and Chetty, 1999; Harbour and
the plates were incubated for additional 4 h at 37°C. At 48 h, 50
cells (Nunc, Denmark). After exposure to various concentrations of atRA for
200 h, 200 cells were aspirated off. To achieve solubilization of the formazan crystal formed in viable
cells, 100 lM MTT solution (Sigma) (2 mg/ml in PBS) was added to each well, and
DMSO was added to each well. The absorbance was read at 540 nm on
a Dias automatic microwell plate reader with DMSO as the blank.

Flow cytometry. Cell cycle distribution and subdiploid fraction were
determined by staining DNA with PI (propidium iodide) (Sigma). Briefly, 10^6 cells were seeded in a 6-well plate and incubated with given concentrations
of atRA for 72 h. Cells were then washed in PBS, fixed in 70% ethanol overnight, after which they were resuspended in PBS containing 400 
μg/ml PI, 0.1 mg/ml RNase (Sigma), and 0.1% Triton-X-100 at 37°C for 30 min. Then
the cells were centrifuged and washed three times with PBS, and the pellets were digested
in 0.5 M NaOH for 30 min with frequent agitation. The reaction was stopped by addition of 8
μl sample buffer and boiling for 5 min. The absorbance was read at 490 nm on
a Dias automatic microwell plate reader with DMSO as the blank.

Western blot analysis. After MEPM cells were seeded in 75 ml flasks and
treated with atRA for 72 h, the cells were harvested and lysed in a buffer containing
100 mM Tris-HCl, pH 6.8; 10% glycerol; 2% sodium dodecyl sulfate (SDS); and
5% β-mercaptoethanol. Then 25 μg protein lysates were separated by 12.5%
SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto nitrocellulose. After blocking in a 5% non-fat dry milk solution in washing buffer
containing 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween-20,
membranes were incubated overnight at 4°C with different antibodies: rabbit
polyclonal anti-cyclin D (Santa Cruz), rabbit polyclonal anti-cyclin E (Santa Cruz), rabbit polyclonal antibody recognizing phospho-Rb-Ser795 (Cell Signaling
Technology), and rabbit polyclonal anti-β-actin (Santa Cruz). After they
were washed three times with Tween-20-BSA, membranes were incubated for
2 h with horseradish peroxidase–coupled secondary antibodies at room temperature.
Signals were detected with the ECL kit (Amersham Pharmacia Biotech).

MATERIALS AND METHODS

Animals. Mature male and female ICR mice were housed at a temperature
of 22°C with a 12 h light/dark cycle. Animals were maintained on Purina mouse
chow and water ad libitum. Matings were accomplished by placing 1 male and
3 females together at 21:00 and allowing them to mate overnight. The presence of
a vaginal plug the following morning was taken as evidence of mating (gestation
day 0; GD 0).

Cell culture. Pregnant mice were euthanized on GD 13, a critical stage of
murine secondary palate development. Embryos were removed from pregnant
dams. The palate shelves were dissected in sterile, cold phosphate-buffered saline
(PBS) and were pooled, minced, and converted into single cell suspensions by
incubating with 0.25% trypsin/0.05% EDTA in PBS for 10 min at 37°C with
constant shaking. Digested samples were briefly triturated and filtered through
70-μm mesh, and cells were seeded on cell-culture dishes and grown to confluence in
DME medium (Gibco BRL) containing 5% fetal calf serum (FBS, Hyclon Co.)
at 37°C in a 95% air/5% CO2 atmosphere, with media replaced every other day.

Trypan blue exclusion assay. Cells were seeded at an initial concentration
of 2 × 10^3 cells/ml in 24-well tissue culture plates, and atRA was added to a
series of concentrations (0, 0.1, 1, 5, and 10 μM, respectively). The final
concentration of vehicle ethanol in all treatments did not exceed 0.1% (v/v) in the
treatment. These events might account for the pathogenesis of cleft palate induced by retinoic acid.

RESULTS

atRA Inhibited MEPM Cell Growth

The effects of atRA on the growth of MEPM cells were
analyzed with the trypan blue dye exclusion test. atRA inhibited
the growth of MEPM cells dose-dependently, ranging from 0.1
to 10 μM (Fig. 1), and showed significant inhibition at
concentrations of 5 and 10 μM after atRA treatment for 24 h, 48 h,
and 72 h (P < 0.05).

FIG. 1. Effect of atRA on the growth of MEPM cells assayed by
trypan blue dye exclusion test. Cells were cultured for 72 h with atRA
at concentrations of 0 (control), and 0.1 to 10 μM. atRA inhibited the growth
of MEPM cells dose-dependently. Values represent means ± S.D. of quad-
truplicate cultures.
Effect of atRA on Cell Viability in MEPM Cells

In this set of experiments, we evaluated the effects of atRA on cell viability in MEPM cells. Cells were treated with different concentrations of atRA for 72 h, and cell viability was determined by MTT assay. A significant inhibition in cell viability was observed after treatment with atRA (Fig. 2). Indeed, 5 and 10 μM atRA inhibited cell viable numbers by 42.6% and 54.8%, respectively, compared with control cells that were treated only with vehicle (indicated as 0 μM).

atRA-Induced Cell Cycle Arrest and Apoptosis in MEPM Cells

In light of the fact that proliferation of MEPM cells was inhibited by high concentrations of atRA, we next used flow cytometry to determine where in the cell cycle they accumulate and whether apoptotic events occurred with them in response to atRA. As shown in Figure 3, we found that in control (vehicle-treated) MEPM cells, 53% of the cells were in G0/G1, 29% in S phase, and 18% in G2/M phase. Upon given concentrations of atRA treatment, an increase in the number of cells in G0/G1 was observed in a dose-responsive manner. Indeed, at the concentration of 10 μM, approximately 80% of cells were in G0/G1, whereas the numbers of cells in S and G2/M were reduced to 12% and 8%, respectively (Fig. 3). Thus, atRA treatment leads to G0/G1 arrest in MEPM cells in a dose-dependent manner. At concentrations of 5 μM and 10 μM, atRA treatment induced the appearance of a sub-G1 fraction (17% and 26%, respectively), which is characteristic of apoptosis.
Effect of atRA on Expression of Cell Cycle Regulatory Molecules

We next determined whether atRA affected expression of molecules that are known to regulate entry of cells into the S phase and thereby play key roles in MEPM cell growth, inhibition, and cell cycle progression. These include cyclins D and E, which govern phosphorylation of Rb, the release of E2F, and the control of exit from G_1 (Sherr, 1996). As shown in Figure 4, atRA treatment decreased the expression levels of cyclin D1 and E in a dose-responsive manner. When we evaluated the levels of phosphorylated Rb with a specific anti-phospho-Rb antibody (phospho Ser 795), we observed a dose-dependent inhibition of phosphorylation of this critical regulator of G_1/S progression.

Alterations in Cell-Cycle Enzyme Activity of cdk2 and cdk4

To further understand the mechanism by which atRA inhibit Rb phosphorylation, we examined their effect on the activity of cdk2 and cdk4. cdk4 is active during early-to-mid G_1 phase and Cdk2 is active in late G_1 phase; both were required for progression through the G_1/S transition through phosphorylating a critical serine residue of Rb (Ser 795) (Koff et al., 1992; Rosenblatt et al., 1992; Swanton, 2004; van den Heuvel and Harlow 1993). The present data show that within the range of concentrations from 0.1 mM to 10 mM, atRA inhibited reaction activity of both cdk2 and cdk4 (Fig. 5).

DISCUSSION

The physiologic doses (usually <0.01 mM) of RA play a important role in embryogenesis. The unsuitable maternal use of certain retinoid-containing medications and excessive supplements at specific critical periods of facial morphogenesis could lead to aberrant development and subsequent cleft palate. The greatest success in the RA-induced cleft palate in mice was demonstrated by a single dose of exogenous atRA on GD 11 at a level of 100 mg/kg. Given the absence of maternal toxicity, it is...
evident that all pharmacological doses (≥1 µM) of RA are at the toxic thresholds. To gain insight into possible mechanisms of atRA-induced cleft palate, we mainly studied the effects of pharmacologic (1 µM) doses of atRA on growth of MEPM cells.

In atRA-treated MEPM cultures, we found a dose-dependent survival suppression that blocked cell cycle progression at the G1/S transition, ultimately leading to G1/G0 arrest and eliciting apoptosis. Data here are in agreement with a previous in vivo study, which showed that the inhibition of growth and excess apoptosis of MEPM cells contributed to the formation of cleft palate and other orofacial congenital malformations (Suwa et al., 2001). It is well known that atRA treatment induced decrease of cell viability and increase of apoptotic phenomena in diverse tumor cell types, including cancers of the breast, lung, liver, and blood (Guzey et al., 1998; Hsu et al., 2001; Jimenez-Lara et al., 2004; Mangiarotti et al., 1998). Some teratogenic effects of retinoids may be due to their ability to inhibit cell growth and induce apoptosis. For example, RA treatment of midgestation mouse embryos induces excess limb bud apoptosis, resulting in malformed limbs (Jiang and Kochhar, 1992). Cleft palate results from failure of fusion between the palatal shelves. One possible cause is retarded mesenchymal proliferation and differentiation in those shelves, which will lead to retardation in the development of the palatal bones (Furukawa et al., 2004; Stark, 1954).

Proliferating mammalian cells pass through several cell cycle checkpoints, mainly G1-to-S and G2-to-M transitions. Three important targets have emerged as likely candidates for effectors of this outcome including the expression of cyclin D1 and E and the phosphorylated status of Rb. Data here show that atRA induced a dose-dependent decrease in cyclins D and E. During early G1 phase, cyclins D bind to and activates cdk4, cyclin D–cdk4 complexes phosphorylate their primary target protein, the tumor suppressor retinoblastoma (Rb) protein. In late G1, cyclins E interact with and activate cdk2. Cyclin E–Cdk2 complexes complete this phosphorylation (Duman-Scheel et al., 2002; Yamada et al., 2004). Hyperphosphorylated Rb no longer binds to its target, E2F, thereby allowing E2F to activate the transcription of genes required for S phase in several consecutive steps and thereby ultimately relieving Rb-mediated repression from S phase, as Rb becomes dephosphorylated just prior to M/G1 transition (Bai et al., 2003; DiPietrantonio et al., 1998; Johnson and Schneider-Broussard, 1998; Sherr, 2000). Given the importance of Rb, it is reasonable that inhibition of phosphorylation of Rb is thought to be one of the important mechanisms by which retinoids inhibit proliferation in MEPM cells.

The formation of secondary palate involves several ordered steps, starting with shelf growth and elevation, and followed by contact, adhesion, and disappearance of the medial edge epithelium. Disruption at any stage of palate development could potentially cause cleft palate. It has been known for some time that maternal treatment of atRA produces embryonic palatal shelves deficient in mesenchymal tissue (Horie and Yasuda, 2001; Ikemi et al., 2001) and exhibiting a decreased rate of 3H-thymidine uptake, indicative of inhibition of cell proliferation (Watanabe et al., 1990). Retinoic acid has also been shown to inhibit proliferation of, and extracellular matrix synthesis in mouse fetal palate mesenchymal cells. These data suggest that perturbation of the process of cell proliferation might account for the formation of small palatal shelves and the failure of palatal fusion in vivo (Watanabe et al., 1988).

Together, these results are consistent with the hypothesis that retinoid-induced cleft palate might depend in part on the effect of RA on growth inhibition and modification of cell cycle distribution of palate mesenchymal cells in the developing palatal shelves. We proposed that decreased cdk activity, followed by reduced phosphorylation of Rb, may account for the failure of palate fusion exposed to RA as the elements of the secondary palate are just beginning to make contract.

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REFERENCES


