TOXICOLOGICAL SCIENCES **89(2)**, 504–509 (2006)
doi:10.1093/toxsci/kfj046
Advance Access publication November 16, 2005

Apoptosis Induced by atRA in MEPM Cells Is Mediated through Activation of Caspase and RAR

Zengli Yu,*,†,‡ Jing Han,† Jiuxiang Lin,*,[1] Ying Xiao,† Xingzhong Zhang,* and Yong Li†,[1]

School of Stomatology, Peking University Health Science Center, Beijing, China; †School of Public Health, Peking University Health Science Center, Beijing, China; and ‡School of Public Health, Zhengzhou University, China

Received September 17, 2005; accepted November 3, 2005

We have previously demonstrated that all-trans retinoic (atRA) induced growth inhibition and apoptosis in mouse embryonic palate mesenchymal cells (MEPM). In the present study, we investigated the molecular mechanisms of atRA-induced apoptosis and its putative action pathway. atRA-induced apoptosis is associated with activation of the initiator caspase-9 and the effector caspase-3, but not of the effector caspase-8. A broad caspase inhibitor (z-VAD-fmk), caspase-9 inhibitor z-LEHD-fmk and caspase-3 inhibitor (z-DEVD-fmk) blocked atRA-induced DNA fragmentation and sub-G1 fraction, but not caspase-8 inhibitor z-IETD-fmk. We further showed that atRA dose-dependently promoted mRNA expression of retinoic acid receptor β (RAR-β) and γ. A weaker increase in RAR-α mRNA was seen only at the highest concentration of atRA (5 μM). The pan RAR antagonist, BMS493, completely abrogated atRA-induced DNA fragmentation, Sub-G1 fraction, and caspase-3 activation. Taken together, these findings show that caspase-mediated induction of apoptosis by atRA is an RAR-dependent signaling pathway.

Key Words: **all-trans retinoic acid; mouse embryonic palatal mesenchymal cells; apoptosis; caspase; retinoic acid receptor.**

The physiologic doses (usually <0.01 μM) of retinoic acid (RA) play an important role in embryogenesis. All-trans RA (atRA), an oxidative metabolite of vitamin A, is also indispensably involved in cell growth and differentiation, reproduction, and embryonic development. However, abnormally high concentrations of RA in both experimental animals (Campbell *et al.*, 2004) and humans (Lammer *et al.*, 1985) result in various fetal malformations (Kamm *et al.*, 1984; Schneider *et al.*, 2001). The greatest success in the RA-induced cleft palate in mice was demonstrated by a single dose exogenous atRA on gestation day 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. Although the teratogenic effects of RA have been well studied, considerable uncertainty remains regarding the specific molecular mechanisms through which it disrupts development. In addition, extensive research in many laboratories worldwide has characterized the receptor-mediated pathway through which retinoid compounds produce their biological responses (Elmazar and Nau, 2004; Elmazar *et al.*, 1997; Matt *et al.*, 2003).

Of the intracellular retinoid binding proteins, nuclear receptors are thought to mediate most of RA's effects on cell behavior. Two subfamilies of nuclear retinoid receptors exist: the RA receptors (RARs) and the retinoid X receptors (RXRs). Within each subfamily there are three subtypes (α, β, and γ) (Mitchell *et al.*, 2003; Jiang *et al.*, 2005). Either atRA or 9-cis RA activates RARs, whereas only 9-cis RA activates RXRs. After ligand binding, these receptors form homodimers or heterodimers and function as transcriptional regulators (Kogai *et al.*, 2005). Both embryonic deletions of individual retinoid receptors and over expression of a constitutively active RAR can induce a number of developmental abnormalities (Lee and Privalsky, 2005; Livera *et al.*, 2001). It has been demonstrated that all-*trans*-retinoic acid and its receptors are present in embryonic tissues in a specific spatial and temporal distribution (Chambon, 1996; Rohwedel *et al.*, 1999; Ross *et al.*, 2000; Yamagata *et al.*, 1994). Additionally, blocking the oxidative metabolism of retinol to all-*trans*-retinoic acid by administration of an alcohol dehydrogenase inhibitor (4-methylpyrazole) significantly lowered the teratogenic response of retinol in mice (Collins *et al.*, 1992). Although the inhibitory-effect of RA in the developing embryonic mesenchyme has been demonstrated, the relationships between RARs and RA-induced growth inhibition are not well understood.

Our previous studies demonstrated that atRA could exert anti-proliferative actions in MEPM cells, which was associated with arrest in the G0/G1 phase and induction of apoptosis (Yu *et al.*, 2005). To further investigate the direct action of atRA at the cellular level, we designed this study to elucidate the molecular mechanisms of atRA-induced apoptosis and its putative action pathway. Our data revealed that atRA-induced

[1] To whom correspondence should be addressed at either Prof. Jiuxiang Lin, School of Stomatology, Peking University Health Science Center, Beijing, 100081, China; Tel./Fax: +86-10-62751207; E-mail: jxlin@pku.edu.cn; or Prof. Yong Li, Laboratory of Molecular Toxicology & Development Molecular Biology, School of Public Health, Peking University Health Science Center, Beijing, 100083, China; Tel./Fax: +86-10-82801177; E-mail: liyong@bjmu.edu.cn.

© The Author 2005. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org

apoptosis in MEPM cells is mediated through activation of caspases and is dependent of RAR pathway.

MATERIALS AND METHODS

Cell culture. Murine embryonic palate mesenchymal (MEPM) cells were derived from palatal tissue from gestational day 13 ICR mouse embryos (date of vaginal plug detection was considered day 0 of gestation). 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 pipetted, filtrated through 70-μm mesh, and cells were seeded on cell-culture dishes and grown to confluence in DMEM medium (Gibco BRL) containing 5% fetal calf serum (FBS, Hyclon Co.) at 37°C in a 95% air/5% CO_2 atmosphere, with media replaced every other day. For experiments cells were treated with various concentrations of atRA. atRA stocks were prepared by dilution with ethanol. Control cells were treated with 0.1% ethanol, which was the maximal final concentration of the vehicle in atRA treated cultures.

Caspase-3, -8, and -9 activity assay. MEPM cells were treated with atRA (0.1–5 μM) and with ethanol vehicle (0.1%, v/v) for 24 h. Analysis of caspase-3, caspase-8, and caspase-9 activities was performed using Caspase Apoptosis Detection Kit (Santa Cruz, CA), according to the manufacturer's procedure. Briefly, cells (1×10^6) were pelleted by centrifugation, washed with PBS two times and incubated in 500 μl lysis buffer on ice for 10 min, then 1×reaction buffer and 10 μl DEVD-AFC (caspase-3), IETD-AFC (caspase-8), or LEHD-AFC (caspase-9) substrates were added to lysis buffer. The reaction mixtures were incubated at 37°C for 60 min. Activities of caspase-3, -8, and -9 were measured by spectrofluorometry. All cultures were done in triplicate. Fluorescence was determined by a Perkin-Elmer L955 fluorimeter at 400 nm excitation and 505 nm emission. Caspase enzymatic activities in cell lysate are directly proportional to the color reaction.

DNA content. Cells (1×10^5) were seeded into 6-well plates and treated with ethanol vehicle (0.1%, v/v) and atRA (0.1–5 μM) for 24 h. Then, the cells were fixed with fixation solution (70% ethanol, 30% PBS) at 4°C overnight. The samples were centrifuged at $800 \times g$ for 10 min and the cell pellets were incubated with DNA staining solution that contained 40 μg/ml propidium iodide (PI, Sigma) and 100 μg/ml RNase A for 30 min in the dark. The cells were analyzed by flow cytometry (Becton Dickinson) and each experiment was repeated three times.

Detection of DNA fragmentations. Nucleosomal DNA fragmentation is characteristic of apoptotic nuclei. Quantification of DNA fragmentation was done using a sensitive, commercial enzyme-immunoassay (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics Corporation) to determine histone-associated-DNA-fragments (mono- and oligo-nucleosomes) created later in apoptosis. This assay detects apoptotic but not necrotic cell death (Frankfurt and Krishan, 2001). Briefly, cells (1×10^4) were seeded into 6-well plates and treated with ethanol vehicle (0.1%, v/v) and atRA (0.1–5 μM) for 24 h. Then, the cells were homogenized in 3 ml of lysis buffer and incubated for 30 min at room temperature. After centrifugation to remove nuclei and cellular debris, the supernatants were diluted 1:2 (v/v) with lysis buffer. Then, 20 μl from each sample were transferred to a 96-well plate pre-coated with antihistone antibody to which 80 μl of immunoreagent mix were added. After incubation and washes, the wells were treated with the chromogen 2,2′-azinobis(3-ethylbinzthiazoline) sulfonic acid as a substrate. The intensity of the color that develops was measured at 405 nm.

Real time quantitative PCR. Total RNA was isolated from atRA-treated MEPM cells using Trizol (Life Technologies). RNA was further purified by using the RNeasy kit (Qiagen). The concentration of extracted RNA from each group was adjusted to 200 ng/μl based on the absorbance value measured at 260 nm. A 25 μl reaction mixture containing 2 μg of total RNA was reverse transcribed to cDNA using oligo dT primers and SuperScript II RT-polymerase (Life Technologies). Real-time quantitative PCR was performed using the ABI PRISM 7700 sequence detector (Applied Biosystems) in combination with SYBRgreen dye. The reaction was performed at 50°C for 2 min, 95 min for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min in triplicate and analyzed using the comparative Ct method according to the TaqMan manual. The sequences of the primers employed in this study were summarized in Table 1, based on the published criteria (Ulven *et al.*, 2000).

TABLE 1
Oligonucleotides Used for RT-PCR Analyses

Gene	Primer sequence	Size (pb)
RAR-α	Sense: 5' CAGTTCCGAAGAGATAGTACC 3' Antisense: 5' TACACCATGTTCTTCTGGATGC 3'	167
RAR-β	Sense: 5' TCGAGACACAGAGTACCAGC 3' Antisense: 5' GAAAAAGCCCTTGCACCCCT 3'	155
RAR-γ	Sense: 5' GCCTCCTCGGGTCTACAAG 3' Antisense: 5' ATGATACAGTTTTTGTCGCGG 3'	155
β-actin	Sense: 5' GGCATCGTGATGGACTCCG 3' Antisense: 5' GCTGGAAGGTGGACAGCG 3'	612

Statistical analysis. All data are presented as mean values ± SD. The data were evaluated by a one-way ANOVA followed by least significant difference (LSD) test as a post hoc test or Dunnett's T3 test. Statistical significance was at $p < 0.05$.

RESULTS

Activation of Caspase-9 and -3 by atRA

Accumulated results indicate that caspases play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli (Shibata *et al.*, 2004). Of which, caspase-3 was classically divided into executioner caspases, and caspase-8 and -9

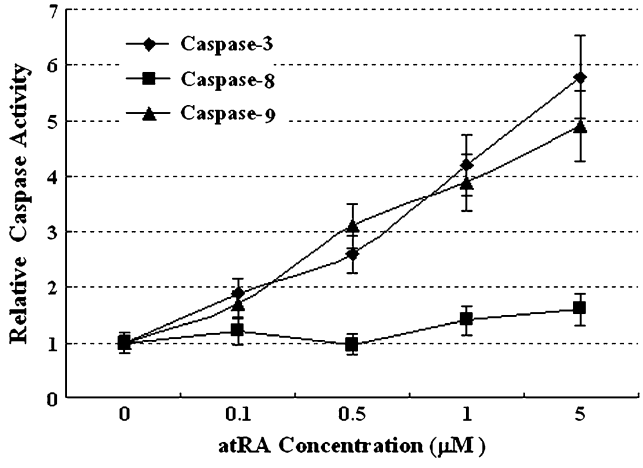

FIG. 1. atRA-induced activation of caspase 3 and -9, but not of caspase-8. MEPM cells were treated with indicated concentrations of atRA for 24 h and activity of caspase was determined using specific fluorescent substrates DEVD-AFC (for caspase 3), IETD-AFC (for caspase 8), or LEHD-AFC (for caspase-9). Data are shown as mean ± SD of three independent experiments.

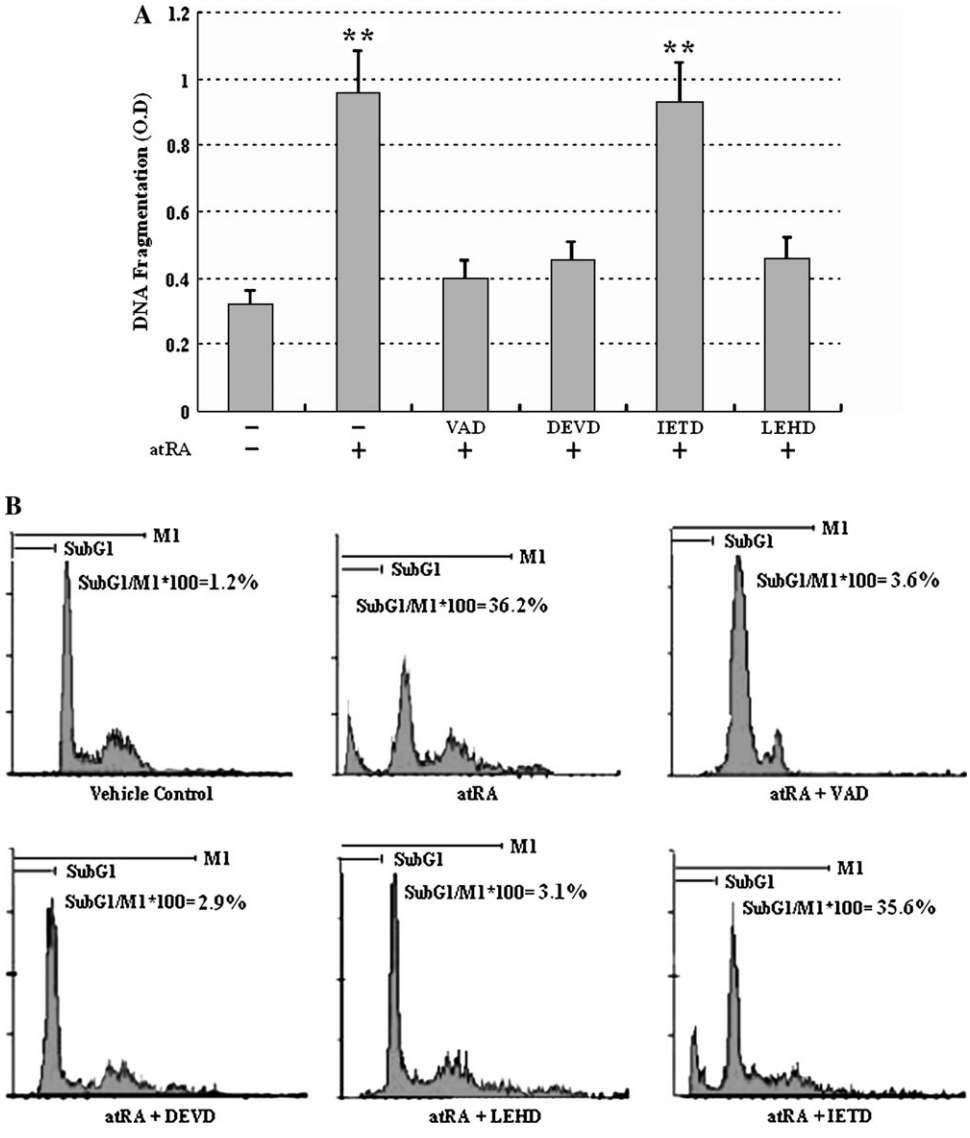

FIG. 2. The effect of caspase inhibitors on atRA-induced apoptosis. MEPM cells were pretreated with the 10 μM of pan caspase inhibitor z-VAD-fmk, caspase-8 inhibitor z-IETD-fmk, caspase-9 inhibitor z-LEHD-fmk or caspase-3 inhibitor z-DEVD-fmk for 1 h, and then exposed to the 5 μM of atRA for another 24 h. (A) DNA fragmentation was determined by the Cell Death Detection ELISA kit. All results were given as means ± SD of triplicate and experiments were repeated twice. $^{**}p < 0.01$ compared to vehicle control cells as analyzed by ANOVA. (B) The percentage of cells in hypodiploid DNA peak (Sub-G1 population) was calculated by Sub-G1 population/total cell cycle populations (M1, Sub-G1 population + G0/G1+S+G2/M) and indicated by numbers shown in each plot.

into initiator caspases according to their function and their sequences of activation (Manabe *et al.*, 2004; Shibata *et al.*, 2004). Thus, we investigated whether caspases were involved in the apoptotic response induced by atRA. As shown in Figure 1, atRA treatment for 24 h caused the activation of caspase-3 and -9 in a dose-response manner, but not of caspase-8.

Activation of Caspase Is Essential for atRA-Induced Apoptosis

To confirm whether activation of caspases is essential for atRA-evoked apoptosis, we next used caspase inhibitors to challenge to block atRA-induced apoptosis. MEPM cells were pretreated with 10 μM of non-selective caspase inhibitor z-VAD-fmk and caspase specific inhibitor z-LEHD-fmk (for caspase-9), z-DEVD-fmk (for caspase-3), and z-IETD-fmk (for caspase-8) for 1 h, and then exposed to 5 μM atRA for another 24 h. The fragmented DNA and Sub-G1 fraction were used as indicators of apoptotic cell death. As shown in Figure 2, the inhibitors of pan caspase, caspase-9 and -3, but not the caspase-8 specific inhibitor, could block atRA-induced DNA fragmentation and Sub-G1 fraction. These results indicate that caspase-9 signaling pathways are involved in atRA-induced apoptosis in MEPM cells.

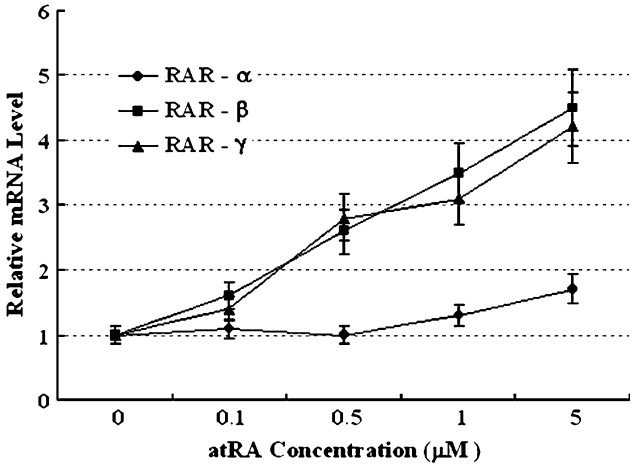

FIG. 3. atRA increased mRNA expression of RAR in MEPM cells. Real-time quantitative PCR was used to quantify the mRNA of RAR in MEPM cells treated with various concentrations of atRA for 24 h. Levels of RAR and β-action expression in each sample were determined by using the relative standard curve method. A relative amount of DNA of RAR was expressed as a ratio to β-action DNA, and the RAR level in vehicle control was set to 1. This experiment was run twice. In each experiment, samples were run in triplicates. Data are expressed as the mean ± SD.

atRA Increased mRNA Expression of RAR

atRA is a non-selective RAR agonist and exerts its biological actions through binding to and activating RAR-α, -β, and -γ. Thus, it was reasonable to speculate that the effects of atRA on the cellular process in MEPM might involve RAR signaling. Real time RT-PCR analyses revealed that atRA dose-dependently stimulated mRNA level of RAR-β and -γ, but only a weaker increase in RAR-α at the highest concentration of atRA (5 μM) (Fig. 3).

atRA-Induced Apoptosis Is Dependent of RAR

In order to further determine the role of RAR in atRA-induced apoptosis, the effect of BMS493, a pan RA receptor antagonist, was studied on atRA-treated MEPM cells. BMS493 had no effect on the basal DNA fragmentation, Sub-G1 fraction and caspase-3 activities in the control cells. When MEPM cells were pretreated with BMS493 and then treated with 5 μM atRA for 24 h, our results clearly indicated that atRA-induced apoptotic indexes were blocked (Fig. 4).

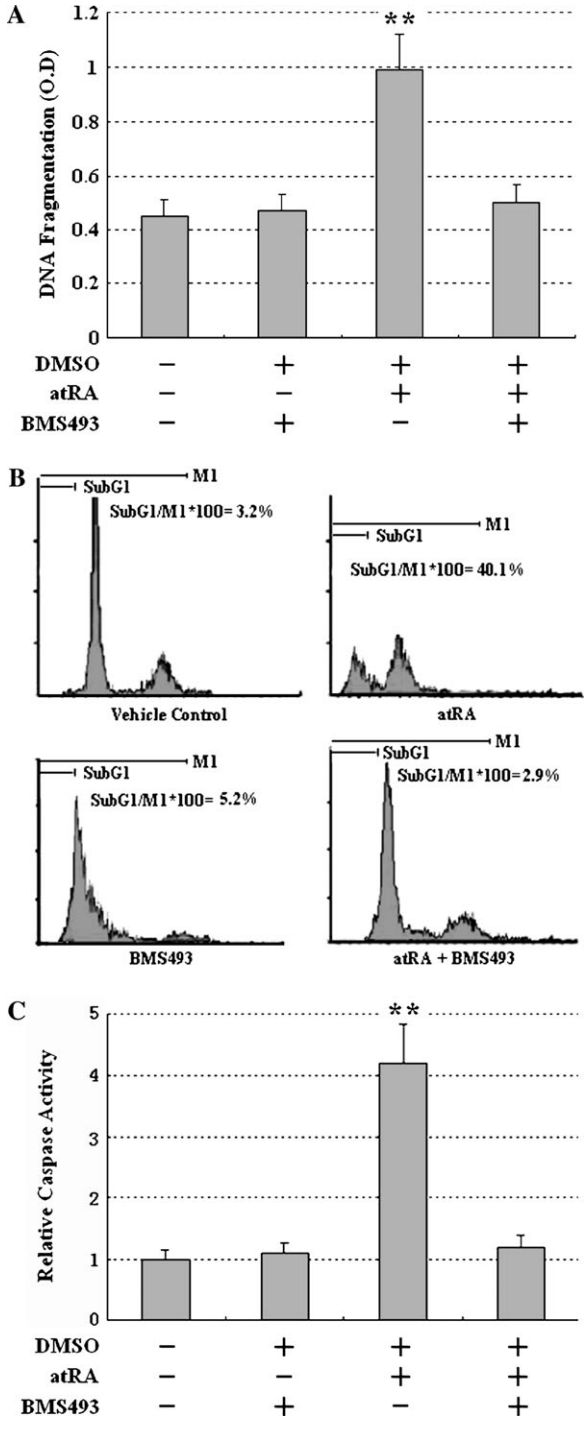

FIG. 4. atRA-induced apoptosis is dependently of RAR. MEPM cells were preincubated for 20 min with or without 3 μM BMS493 and then treated with or without atRA (5 μM) for 24 h. (A) DNA fragmentation was determined by the Cell Death Detection ELISA kit. (B) The percentage of cells in hypodiploid DNA peak (Sub-G1 population) was calculated by Sub-G1 population/total cell cycle populations (M1, Sub-G1 population + G0/G1+S+G2/M) and indicated by numbers shown in each plot. (C) Caspase-3 activity was determined using specific fluorescent substrates DEVD-AFC. All results were given as means ± SD of triplicate and experiments were repeated twice. $^{**}p < 0.01$ compared to vehicle control cells as analyzed by ANOVA.

DISCUSSION

Apoptosis is mediated through two main pathways, the extrinsic (death receptor) pathway (Krammer, 2000) and the intrinsic (mitochondrial) pathway (Kroemer and Reed, 2000). The extrinsic pathway is initiated by ligation of plasma membrane death receptors such as CD95/Fas, TNF receptor, or the TRAIL receptor. Through adapter proteins the receptor

interacts with and activates caspase 8. Activated caspase 8 cleaves and activates effector caspases, with caspase 3 being the major effector caspase (Johnstone *et al.*, 2002), bypassing the mitochondrial apoptotic pathway. The intrinsic pathway involves the disruption of the mitochondrial membrane resulting in the release of mitochondrial proteins including Smac/DIABLO, HtRA2, and cytochrome *c* (Letai *et al.*, 2002; Suzuki *et al.*, 2001; Wang, 2001). Once in the cytosol, cytochrome *c* interacts with Apaf-1 to induce the activation of caspase 9. In turn, activation of the latter causes activation of caspase-3 and -7, which drive the biochemical execution of apoptosis (Cai *et al.*, 1998; Zou *et al.*, 1997).

Our current study demonstrated that atRA-induced apoptosis in MEPM cells was caspase-dependent, since the pan-caspase inhibitor Z-VAD-fmk effectively abrogated its apoptotic actions. Furthermore, caspase-3 and -9 were markedly activated in a dose-dependent manner, and use of the caspase-9 inhibitor (z-LEHD-fmk) or caspase-3 inhibitor (z-DEVD-fmk) could significantly attenuate atRA-induced apoptosis when compared to atRA-treated cultures without inhibitor ($p < 0.01$). In contrast, atRA treatment had no marked effect on caspase-8 activity and caspase-8 specific inhibitor Z-IETD-fmk failed to block atRA-induced apoptosis. The current data herein are consistent with caspase-9 pathway, however, further investigation is necessary to determine a mechanism by which atRA induces the release of cytochrome c from mitochondria.

The actions of RA are ultimately thought to be mediated through specific nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors. Multiple retinoic acid receptors have been identified; among these are RAR-α, β, and γ. RAR-α is expressed ubiquitously in adult tissue and RAR-γ is primarily expressed in skin. RAR-β is unique because it is primarily expressed in epithelial cells and exhibits induced expression in response to retinoic acid (atRA) that is mediated by an enhancer element, the retinoic acid response element (RARE), within its promoter. Also, it has been demonstrated that all-*trans*-retinoic acid and its receptors are present in embryonic tissues in a specific spatial and temporal distribution (Chambon, 1996; Rohwedel *et al.*, 1999; Ross *et al.*, 2003; Yamagata *et al.*, 1994) and extensive research in many laboratories worldwide has characterized the receptor-mediated pathway through which retinoid compounds produce their biological responses (Monczak *et al.*, 1997).

Another question of the present study was to determine the role of RAR in atRA-induced apoptosis. Data revealed that, within the tested range of concentrations, atRA treatment increased mRNA expression of RAR-β and RAR-γ with a dose-dependent manner. Only a weaker induction of RAR-α was seen at the highest concentration of 5 μM atRA. The pan antagonist, BMS493, could completely abrogate atRA-induced DNA fragmentation, Sub-G1 fraction, and caspase-3 activation, suggesting that atRA-induced apoptosis involved the RAR pathway. Many studies have suggested a critical role for RAR in modulating the growth and survival of various cancer cells (Chen *et al.*, 2004; Zang *et al.*, 2003). For example, stable expression of RAR-β in RAR-β-negative PC-3 cells increases their sensitivity to growth inhibition by agonistic retinoids (Campbell *et al.*, 1998). RAR-β and RAR-γ clearly do play some roles in the inhibitory-effects of atRA on cell proliferation and tissue differentiation in the developing embryo (Koyama *et al.*, 1999; Mic *et al.*, 2003). Since endogenous RA is essential for normal embryonic development (Lee *et al.*, 2004; Yashiro *et al.*, 2004), signaling systems that impinge on the RA signal transduction pathway by modulating the expression of its components are likely to play a critical role in the control of organogenesis and development. atRA adversely affected RAR-β and RAR-γ, which might account for its teratogenic effect. In addition, the modest up-regulation of RAR-α by atRA might also play a role in inhibitory effects of atRA. However, it is necessary to carry out further studies to determine the specific role in teratogenic actions played by various RAR isoforms.

In summary, the current study demonstrated that atRA-induced cell death of MEPM was mediated through the caspase-9-dependent intrinsic apoptotic pathway. Also, the RAR activation might play a critical role in this cellular process, since normal palate growth requires a developmental, age-dependent increase in cell numbers. The inhibitory effects of atRA in MEPM cells might be closely relevant to the pathogenesis of atRA-induced cleft palate.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation No. 30371224 and 30572067, People's Republic of China and the Major Basic Research Development Program of People's Republic of China (2001CB510305).

REFERENCES

Cai, J., Yang, J., and Jones, D. P. (1998). Mitochondrial control of apoptosis: The role of cytochrome c. *Biochim. Biophys. Acta* **1366,** 139–149.

Campbell, J. L. Jr., Smith, M. A., Fisher, J. W., and Warren, D. A. (2004). Dose-response for retinoic acid-induced forelimb malformations and cleft palate: A comparison of computerized image analysis and visual inspection. *Birth Defects Res. B Dev. Reprod. Toxicol.* **71,** 289–295.

Campbell, M. J., Park, S., Uskokovic, M. R., Dawson, M. I., and Koeffler, H. P. (1998). Expression of retinoic acid receptor-beta sensitizes prostate cancer cells to growth inhibition mediated by combinations of retinoids and a 19-nor hexafluoride vitamin D3 analog. *Endocrinology* **139,** 1972–1980.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940–954.

Chen, C. F., Goyette, P., and Lohnes, D. (2004). RARgamma acts as a tumor suppressor in mouse keratinocytes. *Oncogene* **23,** 5350–5359.

Collins, M. D., Eckhoff, C., Chahoud, I., Bochert, G., and Nau, H. (1992). 4-Methylpyrazole partially ameliorated the teratogenicity of retinal and reduced the metabolic formation of all-trans-retinoic acid in the mouse. *Arch. Toxicol.* **66,** 652–659.

Elmazar, M. M., and Nau, H. (2004). Potentiation of the teratogenic effects induced by coadministration of retinoic acid or phytanic acid/phytol with synthetic retinoid receptor ligands. *Arch. Toxicol.* **78,** 660–668.

Elmazar, M. M., Ruhl, R., Reichert, U., Shroot, B., and Nau, H. (1997). RARalpha-mediated teratogenicity in mice is potentiated by an RXR agonist and reduced by an RAR antagonist: Dissection of retinoid receptor-induced pathways. *Toxicol. Appl. Pharmacol.* **146,** 21–28.

Frankfurt, O. S., and Krishan, A. (2001). Enzyme-linked immunosorbent assay (ELISA) for the specific detection of apoptotic cells and its application to rapid drug screening. *J. Immunol. Methods* **253,** 133–144.

Jiang, S. Y., Wu, M. S., Chen, L. M., Hung, M. W., Lin, H. E., Chang, G. G., and Chang, T. C. (2005). Identification and characterization of the retinoic acid response elements in the human RIG1 gene promoter. *Biochem. Biophys. Res. Commun.* **331,** 630–639.

Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002). Apoptosis: A link between cancer genetics and chemotherapy. *Cell* **108,** 153–164.

Kamm, J. J., Ashenfelter, K. O., and Ehmann, C. W. (1984). Preclinical and clinical toxicology of selected retinoids. In *The Retinoids*, 2nd ed. (M. B. Sporn, A. B. Roberts, and D. S. Goodman, Eds.), pp. 287–326. Academic Press, Orlando, FL.

Kogai, T., Kanamoto, Y., Li, A. I., Che, L. H., Ohashi, E., Taki, K., Chandraratna, R. A., Saito, T., and Brent, G. A. (2005). Differential regulation of sodium/iodide symporter (NIS) gene expression by nuclear receptor ligands in MCF-7 breast cancer cells. *Endocrinology* **146,** 3059–3069.

Koyama, E., Golden, E. B., Kirsch, T., Adams, S. L., Chandraratna, R. A., Michaille, J. J., and Pacifici, M. (1999). Retinoid signaling is required for chondrocyte maturation and endochondral bone formation during limb skeletogenesis. *Dev. Biol.* **208,** 375–391.

Krammer, P. H. (2000). CD95's deadly mission in the immune system. *Nature* **407,** 789–795.

Kroemer, G., and Reed, J. C. (2000). Mitochondrial control of cell death. *Nat. Med.* **6,** 513–519.

Lammer, E. J., Chen, D. T., Hoar, R. M., Agnish, N. D., Benke, P. J., Braun, J. T., Curry, C. J., Fernhoff, P. M., Grix, A. W., Lott, I. T., *et al.* (1985). Retinoic acid embryopathy. *N. Engl. J. Med.* **313,** 837–841.

Lee, G. S., Kochhar, D. M., and Collins, M. D. (2004). Retinoid-induced limb malformations. *Curr. Pharm/Des.* **10,** 2657–2699.

Lee, S., and Privalsky, M. L. (2005). Heterodimers of retinoic acid receptors and thyroid hormone receptors display unique combinatorial regulatory properties. *Mol. Endocrinol.* **19,** 863–878.

Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* **2,** 183–192.

Livera, G., Rouiller-Fabre, V., and Habert, R. (2001). Retinoid receptors involved in the effects of retinoic acid on rat testis development. *Biol. Reprod.* **64,** 1307–1314.

Manabe, N., Goto, Y., Matsuda-Minehata, F., Inoue, N., Maeda, A., Sakamaki, K., and Miyano, T. (2004). Regulation mechanism of selective atresia in porcine follicles: Regulation of granulosa cell apoptosis during atresia. *J. Reprod. Dev.* **50,** 493–514.

Matt, N., Ghyselinck, N. B., Wendling, O., Chambon, P., Mark, M. (2003). Retinoic acid-induced developmental defects are mediated by RARbeta/RXR heterodimers in the pharyngeal endoderm. *Development* **130,** 2083–93.

Mic, F. A., Molotkov, A., Benbrook, D. M., and Duester, G. (2003). Retinoid activation of retinoic acid receptor but not retinoid X receptor is sufficient to rescue lethal defect in retinoic acid synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 7135–7140.

Mitchell, L. E., Murray, J. C., O'Brien, S., and Christensen, K. (2003). Retinoic acid receptor alpha gene variants, multivitamin use, and liver intake as risk factors for oral clefts: A population-based case-control study in Denmark, 1991–1994. *Am. J. Epidemiol.* **158,** 69–76.

Monczak, Y., Trudel, M., Lamph, W. M., and Miller, W. H. (1997). Induction of apoptosis without differentiation by retinoic acid in PLB-985 cells requires the activation of both RAR and RXR. *Blood.* **90,** 3345–3355.

Rohwedel, J., Guan, K., and Wobus, A. M. (1999). Induction of cellular differentiation by retinoic acid *in vitro*. *Cells Tissues Organs* **165,** 190–202.

Ross, S. A., McCaffery, P. J., Drager, U. C., and De Luca, L. M. (2000). Retinoids in embryonal development. *Physiol. Rev.* **80,** 1021–1054.

Schneider, R. A., Hu, D., Rubenstein, J. L., Maden, M., and Helms, J. A. (2001). Local retinoid signaling coordinates forebrain and facial morphogenesis by maintaining FGF8 and SHH. *Development* **128,** 2755–2767.

Shibata, M. A., Morimoto, J., Ito, Y., Kusakabe, K., and Otsuki, Y. (2004). Experimental gene therapy in mammary and urinary bladder cancer using electrogene transfer. *Med. Electron. Microsc.* **37,** 216–224.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell.* **8,** 613–621.

Ulven, S. M., Gundersen, T. E., Weedon, M. S., Landaas, V. O., Sakhi, A. K., Fromm, S. H., Geronimo, B. A., Moskaug, J. O., and Blomhoff, R. (2000). Identification of endogenous retinoids, enzymes, binding proteins, and receptors during early postimplantation development in mouse: important role of retinal dehydrogenase type 2 in synthesis of all-trans-retinoic acid. *Dev. Biol.* **220,** 379–391.

Wang, X. (2001). The expanding role of mitochondria in apoptosis, *Genes Dev.* **15,** 2922–2933.

Yamagata, T., Momoi, M. Y., Yanagisawa, M., Kumagai, H., Yamakado, M., and Momoi, T. (1994). Changes of the expression and distribution of retinoic acid receptors during neurogenesis in mouse embryos. *Brain. Res. Dev. Brain. Res.* **77,** 163–176.

Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y., and Hamada, H. (2004). Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. *Dev. Cell.* **6,** 411–422.

Yu, Z., Lin, J., Xiao, Y., Han, J., Zhang, X., Jia, H., Tang, Y., and Li, Y. (2005). Induction of cell-cycle arrest by all-trans retinoic acid in mouse embryonic palatal mesenchymal (MEPM) cells. *Toxicol. Sci.* **83,** 349–354.

Zang, C., Wachter, M., Liu, H., Posch, M. G., Fenner, M. H., Stadelmann, C., von Deimling, A., Possinger, K., Black, K. L., Koeffler, H. P., *et al.* (2003). Ligands for PPARgamma and RAR cause induction of growth inhibition and apoptosis in human glioblastomas. *J. Neurooncol.* **65,** 107–118.

Zou, H., Henzel, W. J., Liu, X., Lutschg, A., Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell.* **90,** 405–413.