Selective Roles of Retinoic Acid Receptor and Retinoid X Receptor in the Suppression of Apoptosis by All-trans-retinoic Acid*

Received for publication, December 6, 2000
Published, JBC Papers in Press, January 16, 2001, DOI 10.1074/jbc.M011000200

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Vitamin A (retinol) and its biologically active derivatives retinoids exert a wide range of biological effects on embryogenesis, neoplasia, and maintenance of normal tissues, especially epithelium (1). The crucial roles of retinoids in controlling cell function have been elucidated especially using retinoid acid (RA),1 the most notable retinoid. Previous reports showed that RA has profound inhibitory effects on tumorigenesis. It is via suppression of cell growth and induction of cellular differentiation (1). It has also been reported that RA induces apoptosis of various tumor cells in vitro (2, 3), and it may contribute to the antitumor effect of RA. However, the mode of action of RA on apoptosis is a little complicated. We recently reported that all-trans-RA (t-RA) inhibited H2O2-triggered apoptosis in mesangial cells and fibroblasts (4). The molecular mechanisms involved in the antiapoptotic effect of t-RA are not fully elucidated, but activator protein 1 (AP-1) is a possible target. It is because 1) the AP-1 pathway plays a crucial role in the H2O2-induced apoptosis (5, 6) and 2) t-RA is a known inhibitor of AP-1 in some cell types (7).

AP-1, mainly composed of heterodimers of c-Jun and c-Fos, binds to the particular cis element, 12-O-tetradecanoylphorbol-13-acetate response element, and initiates transcription of target genes (8). Several molecular events are involved in the activation of AP-1; i.e. the transacting potential of AP-1 depends on induction and phosphorylation of AP-1 components by mitogen-activated protein (MAP) kinases (9). For example, expression of c-fos is regulated by ternary complex factors whose activity is regulated by extracellular signal-regulated kinase, p38 MAP kinase, and c-Jun N-terminal kinase (JNK). Expression of c-jun is regulated by c-Jun and activating transcription factor-2, which are phosphorylated by JNK and/or p38 MAP kinase. Post-translational activation of AP-1 is also regulated by MAP kinase-mediated phosphorylation. c-Jun is phosphorylated and activated by JNK, and c-Fos is phosphorylated by a member of the MAP kinase family, Fos-regulating kinase (9). To elucidate molecular events involved in the antiapoptotic effect of t-RA, we previously investigated the effect of t-RA on the expression of c-fos and c-jun and activation of JNK triggered by H2O2. Our data showed that t-RA inhibited AP-1 activation and that it was associated with suppression of both c-fos/c-jun expression and JNK phosphorylation (4).

The biological actions of RA, especially its transcriptional regulation of target genes, are mediated by two families of nuclear receptors, retinoic acid receptors (RAR-α, -β, and -γ) and retinoid X receptors (RXR-α, -β, and -γ) (10). These receptors have different ligand specificity; e.g. RARs are activated by both t-RA and 9-cis RA, whereas RXRs are activated only by 9-cis RA (10). After the ligand binding, these receptors form homodimers or heterodimers and function as transcriptional regulators. For example, t-RA binds to RARs and activates RAR-RXR heterodimers, and the complex exerts its biological effects via binding to particular cis elements, retinoic acid response elements (11). However, currently, little is known about the requirement of RAR and RXR for the regulation of apoptosis by RA. In this report, we investigated whether RAR and RXR are essential for the antiapoptotic effect of t-RA in H2O2-exposed mesangial cells. We further examined whether
these nuclear receptors are required for the t-RA-mediated suppression of c-fos/c-jun expression, JNK phosphorylation, and AP-1 activation.

EXPERIMENTAL PROCEDURES

Cells—Mesangial cells (SM43) were established from isolated glomeruli of a male Harlan Sprague-Dawley rat and identified as being of the mesangial cell phenotype as described before (12). Cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Life Technologies, Inc.) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 10% fetal calf serum (FCS). Medium containing 1% FCS was generally used for experiments.

Establishment of Stable Transfectants—SM/DRAR8 and SM/DRXR3 were created as follows. Using a modified calcium phosphate coprecipitation method (13), SM43 cells were stably transfected with expression plasmid pRARα-403* (a gift of Dr. R. M. Evans, The Salk Institute, San Diego, CA) or pCDM/RXRΔAF2 (a gift of Dr. J. W. Lee, Chonnam National University, Kwangju, Korea), together with pcDNA3 (Invitrogen, Groningen, The Netherlands). pRARα-403* and pCDM/RXRΔAF2 introduce a dominant-negative form of RAR-α (ΔRAR) and a dominant-negative RXR-α (ΔRXR), respectively. These dominant-negative mutants inhibit all subtypes of RARs or RXRs (14, 15). Stable transfectants were selected in the presence of G418 (750 μg/ml), and clones SM/ΔRAR8 and SM/ΔRXR3 were established. Expression of transgenes was confirmed by Northern blot analysis. A control clone SM/Neo was created by transfection of SM43 cells with pcDNA3 alone.

Pharmacological Manipulation—Confluent cells were pretreated with or without RAR pan-antagonist AGN193109 (5 μM; a gift of Dr. R. A. S. Chandraratna, Allergan Pharmaceuticals, Irvine, CA) (16) or RXR pan-antagonist HX531 (2.5 μM; a gift of Dr. H. Kagechika, University of Tokyo, Tokyo, Japan) (17) for 30 min, treated with or without t-RA (tretinoin, 2 μM; Sigma) for 0.5–2 h and then stimulated...
by H$_2$O$_2$ (150 μM; Sigma) for indicated time periods.

Assessment of Apoptosis—After induction of apoptosis, morphologic examination was performed by phase-contrast microscopy. For fluorescence microscopy analysis, cells were fixed in 4% formaldehyde for 10 min and stained with Hoechst 33258 (10 μg/ml; Sigma) for 1 h. Apoptosis was identified using morphological criteria including shrinkage of the cytoplasm, membrane blebbing, and nuclear condensation and/or fragmentation.

Transient Transfection with Dominant-Negative RAR and RXR—Transient transfection was performed using the calcium phosphate coprecipitation method as described before (18). Cells cultured in 24-well plates were cotransfected with pRSh/RAR($^a$b) or pCDM/RRXΔA2, or a control vector (750 ng/well, respectively), together with pc1-βGal (250 ng/well; a gift of Promega, Madison, WI), pc1-βGal introduces a β-galactosidase gene (lacZ) under the control of the immediate early enhancer/promoter of human cytomegalovirus. After incubation overnight, medium was replaced with 1% FCS. After 24 h, cells were pretreated with t-RA for 30 min, stimulated by H$_2$O$_2$ (175 μM) for 4 h, and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay (13). The percentage of shrunken/rounded blue cells against the total number of blue cells was calculated for each well, and the mean value of four wells was used to compare data in different groups. Assays were performed in quadruplicate.

Reporter Assay—The effect of t-RA on the activity of AP-1 was evaluated by a reporter assay as described before (5, 6). In brief, cells cultured in 24-well plates were transiently transfected with AP-1 reporter plasmid pTRE-LacZ (350 ng/well) or control plasmid pCI-βGal (350 ng/well). pTRE-LacZ introduces lacZ under the control of tandemly repeated AP-1 binding sites (19). After the transfection, cells were incubated in 1% FCS for 36 h, pretreated with AGN193109 or HX531 for 1 h, treated with or without t-RA for 1 h, and then stimulated by H$_2$O$_2$ (125 μM) for 30 h. Relative activity of AP-1 was evaluated as described under “Experimental Procedures.” Data are expressed as means ± S.D. Asterisks indicate statistically significant differences (p < 0.05). Assays were performed in quadruplicate.

Expression by t-RA—To further investigate roles of RAR and RXR in the anti-AP-1 effect of t-RA, reporter assays were performed in quadruplicate.

Northern Blot Analysis—Expression of c-fos, c-jun, RAR-$^a$, and RXR-$^a$ was examined by Northern blot analysis (4). In brief, total RNA was extracted by the single-step method (20) and subjected to analysis. To prepare probes, cDNAs for c-fos (21), c-Jun (22), RAR-$^a$ (23), and RXR-$^a$ (23) (gifts of Dr. S. J. Collins, Fred Hutchinson Cancer Research Center, Seattle, WA) were labeled by the random priming method. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

JNK Assay—Confluent cells cultured in 6-well plates in the presence of 1% FCS for 24 h were pretreated with or without AGN193109 or HX531 for 1 h, treated with t-RA for 2 h, and exposed to H$_2$O$_2$ (200 μM) for 20 min. Activity of JNK was evaluated by the level of phosphorylated JNK, using PhosphoPlus stress-activated protein kinase/JNK (Thr182/Tyr185) antibody kit (Cell Signaling Technology, Beverly, MA) following the protocol provided by the manufacturer.

Statistical Analysis—Data were expressed as means ± S.D. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A p value of < 0.05 was used to indicate a statistically significant difference.

RESULTS

Roles of RAR and RXR in the Anti-AP-1 Effect of t-RA—To examine roles of RAR and RXR in the antiapoptotic effect of t-RA, RAR pan-antagonist AGN193109 and RXR pan-antagonist HX531 were used. Mesangial cells were pretreated with AGN193109 or HX531, treated with t-RA, and then stimulated by H$_2$O$_2$. Phase-contrast microscopy showed that H$_2$O$_2$-treated cells showed nuclear condensation and membrane blebbing typical of apoptosis (Fig. 1A). When pretreated with t-RA, the apoptotic changes were suppressed. The antiapoptotic effect of t-RA was abrogated by the pretreatment with either AGN193109 or HX531 (Fig. 1A). The effects of receptor antagonists were further confirmed quantitatively using Hoechst staining (Fig. 1B). Fluorescence microscopy showed that exposure of cells to H$_2$O$_2$ induced nuclear condensation (% apoptosis, 81.0 ± 7.1%; mean ± S.D.), and t-RA protected the cells from apoptosis (% apoptosis, 4.5 ± 2.9%; p < 0.05). In contrast, the cells pretreated with AGN193109 or HX531 exhibited significant apoptosis even in the presence of t-RA (55.9 ± 10.4% in AGN193109-treated cells; 63.2 ± 12.0% in HX531-treated cells). Treatment with AGN193109 or HX531 alone did not induce any apoptotic changes (data not shown).

The roles of RAR and RXR in the antiapoptotic effect of t-RA were further examined using dominant-negative mutants. Mesangial cells were transiently transfected with ΔRAR or ΔRXR, together with a β-galactosidase gene. When overexpressed, these mutant receptors selectively and effectively inhibit the function of RARs or RXRs (14, 15). After the transfection, cells were pretreated with t-RA and stimulated by H$_2$O$_2$. Consistent with the results using pharmacological inhibitors, dominant-negative inhibition of RAR or RXR significantly reduced the antiapoptotic effect of t-RA (Fig. 1C).

Roles of RAR and RXR in the Anti-AP-1 Effect of t-RA—We previously reported that the AP-1 pathway plays a crucial role in the induction of apoptosis by H$_2$O$_2$ and that t-RA inhibits H$_2$O$_2$-induced apoptosis by suppression of AP-1 (4). To examine roles of RAR and RXR in the anti-AP-1 effect of t-RA, reporter assay was performed. Cells were transfected with an AP-1 reporter plasmid or a control plasmid, pretreated with AGN193109 or HX531, treated with t-RA, and then stimulated by H$_2$O$_2$. As shown in Fig. 2, exposure of cells to H$_2$O$_2$ significantly increased AP-1 activity (183.3 ± 24.0% versus untreated control, 100 ± 19.1%; p < 0.05). Pretreatment with t-RA suppressed the AP-1 activation to 88.2 ± 15.6%. Both AGN193109 and HX531 significantly reversed the attenuated AP-1 activity (154.0 ± 27.4% and 149.7 ± 17.5%, respectively; p < 0.05).

Roles of RAR and RXR in the Suppression of c-fos and c-jun Expression by t-RA—To further investigate roles of RAR and RXR in the anti-AP-1 effect of t-RA, we examined effects of receptor antagonists on the suppression of c-fos and c-jun by t-RA (Fig. 3). Cells were pretreated with AGN193109 or

FIG. 2. Roles of RAR and RXR in the anti-AP-1 effect of t-RA. Cells were transfected with AP-1 reporter plasmid pTRE-LacZ or control plasmid pCI-βGal, pretreated with AGN193109 or HX531 for 1 h, treated with t-RA for 1 h, and then stimulated by H$_2$O$_2$ (125 μM) for 30 h. Relative activity of AP-1 was evaluated as described under “Experimental Procedures.” Data are expressed as means ± S.D. Asterisks indicate statistically significant differences (p < 0.05). Assays were performed in quadruplicate.

FIG. 3. Roles of RAR and RXR in the suppression of c-fos and c-jun expression by t-RA. Cells were pretreated with AGN193109 or HX531 for 30 min, treated with or without t-RA for 30 min, and then stimulated by H$_2$O$_2$ (150 μM) for 2 h. Expression of c-fos and c-jun was examined by Northern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.
Roles of RAR and RXR in the Suppression of JNK Activation by t-RA—We further examined effects of receptor antagonists on the suppression of JNK by t-RA (Fig. 4). Cells were pretreated with AGN193109 or HX531, treated with t-RA, and then stimulated by H₂O₂ for 30 min. Kinase assay showed that stimulation by H₂O₂ induced activation of JNKs (p54 and p46), and treatment with t-RA suppressed JNK activation. Pretreatment with AGN193109 or HX531 failed to reverse the suppressed JNK activity.

The lack of involvement of RAR and RXR was further confirmed using stable transfectants. Mesangial cells were stably transfected with the dominant-negative constructs for ΔRAR and ΔRXR, which were used in the transient transfection study (Fig. 1C). Abundant expression of transgenes in the established SM/ΔRAR8 and SM/ΔRXR3 cells was confirmed by Northern blot analysis (Fig. 5A). Using these clones, activity of JNK was evaluated. Kinase assay revealed that, in response to H₂O₂, JNK was activated similarly in these clones. Overexpression of ΔRAR or ΔRXR did not affect the suppressive effect of t-RA on JNK (Fig. 5B).

DISCUSSION

In this report, we describe the roles of RAR and RXR in the antiapoptotic effect of t-RA on oxidant-induced apoptosis. We found that both nuclear receptors were required for the antiapoptotic effect of t-RA, as well as its inhibitory effect on AP-1, the crucial molecule involved in H₂O₂-induced apoptosis. However, the roles of RAR and RXR in the regulation of individual AP-1 components by t-RA were found to be different. RAR was necessary for the suppressive effect of t-RA on both c-fos and c-jun expression, whereas RXR was required only for its inhibitory effect on c-fos. Furthermore, suppression of JNK activation by t-RA was mediated by neither RAR nor RXR. To our knowledge, this is the first to demonstrate selective roles of RAR and RXR in the t-RA-induced, antiapoptotic pathway.

In general, RAR and RXR are required for various biological effects of RA including transcriptional regulation, induction of apoptosis, growth suppression, and cellular differentiation (1, 10). In some cases, however, RA can also exert its effects without nuclear receptors. For example, t-RA modulates the activity of protein kinase C by direct binding to the retinoic acid binding site of protein kinase C-α (24). Previous reports suggested that the proapoptotic effect of t-RA is mediated by both receptor-dependent (25, 26) and receptor-independent mechanisms (27). However, it has not been determined whether RAR and RXR are involved in the antiapoptotic effect of t-RA. In this report, we elucidated that the antiapoptotic effect of t-RA also involved receptor-dependent and -independent mechanisms. That is, the suppression of c-fos and c-jun by t-RA was mediated by RAR and RXR, which is consistent with a previous report showing that expression of c-fos in response to epidermal growth factor was inhibited by t-RA through an RAR/RXR-dependent pathway (28). In contrast, we found that the inhibition of JNK by t-RA was nuclear receptor-independent. It was confirmed by both pharmacological inhibitors and dominant-negative mutants. This result is contrastive to a previous report that showed receptor-dependent inactivation of JNK by t-RA in serum-stimulated epithelial cells (29). The reason for this discrepancy is currently unclear, but it may be because of differences in stimuli and downstream pathways to JNK activation. Of note, Lee et al. (29) showed that t-RA inhibited JNK activation in a bimodal pattern, i.e. transient activation at early phase and sustained activation at late phase and that nuclear receptors were required for the late but not for the early suppression of JNK.

Currently, the mechanism involved in the RAR/RXR-independent suppression of JNK is not determined. One possibility is that the suppressive effect of t-RA observed here might be mediated by other types of receptors. For example, it has been reported that t-RA can bind to insulin-like growth factor-II receptor and enhances the primary function of this receptor (30). Another previous report showed that the insulin-like growth factor/insulin-like growth factor receptor axis can inhibit the tumor necrosis factor-α-induced JNK activation via the phosphatidylinositol 3-kinase pathway (31). The similar mechanism could be involved in the RAR/RXR-independent suppression of JNK by t-RA.

It is known that t-RA binds to RAR but not to RXR. From this
viewpoint, our finding that RXR inhibitors attenuated the effects of t-RA is interesting. Because t-RA exerts various effects through heterodimerization of RAR and RXR (10, 32), a simple explanation may be that t-RA binds to RAR and facilitates their heterodimerization with RXR, which is essential for its anti-AP-1 action. RXR is supposedly indispensable for the antiapoptotic and anti-AP-1 effects of t-RA. The facts that t-RA-mediated differentiation, growth suppression, and inhibition of c-fos expression required not only RAR but also RXR further support this hypothesis (26, 28).

Another interesting finding in this report is that the suppression of c-jun expression by t-RA was reversed by RAR antagonist but not by RXR antagonist. In contrast to its effect on c-fos, RXR was found to be unnecessary for the suppressive effect of t-RA on c-jun. Expression of c-fos is regulated by ternary complex factors whose activity is regulated mainly by extracellular signal-regulated kinase and p38 MAP kinase. In contrast, expression of c-jun is regulated by c-Jun and activating transcription factor-2 that are activated mainly by JNK (19). The different regulation of c-jun and c-fos by RAR and RXR could be because of different roles of these receptors in the regulation of individual MAP kinases.

Acknowledgments—We appreciate kind gifts of receptor antagonists from Dr. R. A. S. Chandraratna and Dr. H. Kagechika, expression plasmids from Dr. R. M. Evans and Dr. J. W. Lee, and cDNA probes from Dr. S. J. Collins. We also thank Dr. Javier Lucio-Cazana for useful discussion and helpful comments.

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\textit{J. Biol. Chem.} 2001, 276:12697-12701.
doi: 10.1074/jbc.M011000200 originally published online January 16, 2001

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