Suppression of Apoptosis by All-trans-Retinoic Acid

DUAL INTERVENTION IN THE c-JUN N-TERMINAL KINASE-AP-1 PATHWAY*

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Retinoic acid induces apoptosis of various cells, whereas little is known about its anti-apoptotic potential. In this report, we describe an anti-apoptotic property of all-trans-retinoic acid (t-RA) in mammalian cells. Mesangial cells exposed to hydrogen peroxide (H2O2) exhibited shrinkage of the cytoplasm, membrane blebbing, condensation of nuclei, and DNA fragmentation. Pretreatment with t-RA attenuated the morphologic and biochemical hallmarks of apoptosis. t-RA also inhibited apoptosis of mesangial cells triggered by pyrrolidine dithiocarbamate, whereas it did not prevent tumor necrosis factor-α-induced apoptosis. The anti-apoptotic effect against H2O2 was similarly observed in NRK49F fibroblasts, but not in Madin-Darby canine kidney epithelial cells and ECV304 endothelial cells. Mesangial cells exposed to H2O2 undergo apoptosis via the activator protein 1 (AP-1)-dependent pathway. We found that t-RA abrogated the H2O2-induced expression of c-fos/c-jun and activation of AP-1. Furthermore, t-RA inhibited H2O2-triggered activation of c-Jun N-terminal kinase (JNK), and dominant-negative inhibition of JNK attenuated the H2O2-induced apoptosis. These data disclosed the novel potential of retinoic acid as an inhibitor of apoptosis. The anti-apoptotic action of t-RA was ascribed, at least in part, to dual suppression of the cell death pathway mediated by JNK and AP-1.

Apoptosis of glomerular cells is observed in several types of glomerulonephritis (1–4). The molecular mechanisms involved in the apoptotic process have not been identified yet, but several possibilities are postulated. During initiation and progression of inflammation, toxic substances elaborated by leukocytes may induce apoptosis of glomerular cells. Putative triggers include cytokines, nitric oxide, and reactive oxygen intermediates (ROI) (5–8). ROI play crucial roles in the generation of a broad array of human and experimental glomerular diseases (9). Using hydrogen peroxide (H2O2) as a trigger, recent studies have shown that ROI induces apoptosis of glomerular mesangial cells (8, 10, 11).

Multiple signaling cascades may be involved in the H2O2-initiated apoptosis of glomerular cells. Pathways mediated by activator protein 1 (AP-1) are possible candidates. AP-1 is generally regarded as a redox-sensitive transcription factor (12). AP-1, mainly composed of either homodimers of c-Jun or heterodimers of c-Jun and c-Fos, binds to the particular cis element, 12-O-tetradecanoylphorbol-13-acetate response element (TRE), and initiates transcription of target genes (13). Several reports have shown the importance of c-Jun N-terminal kinase (JNK) and its substrate c-Jun in the signaling pathways to apoptosis. For example, exposure of cells to apoptotic stimuli including ultraviolet light, γ-irradiation, tumor necrosis factor-α (TNF-α), and ceramide triggers JNK activity (14–17). Dominant-negative inactivation of SEK1 (JNK kinase), JNK, or c-Jun prevents certain apoptotic processes (14, 15, 17–19). Furthermore, constitutive activation of the JNK-AP-1 pathway results in apoptotic cell death (19–21).

In apoptosis of mesangial cells exposed to H2O2, activation of AP-1 also plays a crucial role. We previously reported that H2O2 induces expression of c-jun and activation of AP-1 (10). Down-regulation of c-Jun/AP-1 using either a dominant-negative mutant of c-Jun, an antisense c-jun, or a pharmacological inhibitor of c-jun attenuated the H2O2-initiated apoptosis (10). Furthermore, suppression of c-jun expression and AP-1 activation by flavonoid quercetin and heparin was closely associated with attenuation of H2O2-induced apoptosis in mesangial cells (11, 22).

Retinoic acid (RA) is an active metabolite of vitamin A and regulates a wide range of biological processes including cell proliferation, differentiation, and morphogenesis (23). The action of retinoids, including RA, is mediated by specific nuclear receptors, namely, retinoic acid receptors (RAR-α, -β, -γ) and retinoid X receptors (RXR-α, -β, -γ). RXRs form homodimers and heterodimers with RARs or other nuclear hormone receptors and function as transcriptional regulators. All-trans-RA (t-RA), for example, activates RAR-RXR heterodimers and exerts its biological actions via binding to particular cis response elements, retinoic acid response elements (24). In certain cell types, RA functions as a potent inhibitor of AP-1 (25). A previous study showed that t-RA inhibited serum-induced activation of AP-1 in mesangial cells (26).

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The effect of t-RA on the activity of AP-1 was evaluated by a transient transfection assay as described before (10, 11). In brief, using the calcium phosphate coprecipitation method, mesangial cells cultured in 24-well plates (1 x 10^5 cells/well) were transiently transfected with an AP-1 reporter plasmid pTRE-LacZ (0.33 μg/well) (34) or a control plasmid pCI-βGal (0.33 μg/well; a gift from Promega, Madison, WI). pTRE-LacZ introduced a β-galactosidase gene (lacZ) under the control of the immediate-early enhancer/promoter of human cytomegalovirus. After transfection, cells were incubated for 48 h in 10% FCS in the presence or absence of t-RA (5 μM) and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay to evaluate AP-1 activity.

RESULTS

Suppression of H_2O_2-induced Apoptosis of Mesangial Cells by t-RA—Rat mesangial cells cultured in the presence of 1% FCS were pretreated with t-RA (5 μM) for 2 h and stimulated by 75–100 μM H_2O_2 for 30 min and stained by Hoechst 33258 (10 μg/ml) for 1 h. Activity of JNK was evaluated by phosphorylation of c-Jun, using the SAPK/JNK Assay Kit (New England Biolabs, Herts, United Kingdom) following the protocol provided by the manufacturer.

Statistical Analysis

All experiments were repeated at least twice. Data were expressed as mean ± S.E. Statistical analysis was performed using the non-parametric Mann-Whitney U test to compare data in different groups. p Value of < 0.05 was used to indicate a statistically significant difference.

Assessment of Apoptosis

Microscopic Analyses—Morphologic examination was performed using a fluorescence microscope. For fluorescence microscopy, cells were fixed with 4% formaldehyde in phosphate-buffered saline for 10 min and stained by Hoechst 33258 (10 μg/ml) for 1 h. Apoptosis was identified using morphological criteria including shrinkage of the cytoplasm, membrane blebbing, and nuclear condensation and/or fragmentation. In contrast to other cell types, MDCK cells undergoing apoptosis easily detach from the substratum. For this cell type, Hoechst analysis was performed using floating cells. To confirm that the major mechanism of cell death induced by H_2O_2, PDTC, and Tnf-α is apoptosis, cells were stained with acridine orange (50 μM) and 10% fetal calf serum (FCS). Medium containing 1% FCS was generally used for experiments.

Nuclear factor-κB (NF-κB)-inactive mesangial cells were created as follows. Sm43 mesangial cells were exposed to diluted retrovirus that introduces a super-repressor mutant of LecB (IeBoM) and a neomycin phosphotransferase gene (28). This retroviral vector was generated by transfection of the helper-free ecotropic packaging line pE19 (29) with pLLeBoMSN (28). Stable infected cells were selected in the presence of G418 (750 μg/ml) and SM/LeBoM cells were established. SM/LeBoM cells exhibit blunted activation of NF-κB in response to interleukin-1β and TNF-α, when examined by electronmicroscopy mobility shift assay (30).

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Pharmacological Manipulation

Mesangial cells (1 x 10^5/well) for 24-well plates; 5 x 10^5/well for 6-well plates) were pretreated with or without t-RA (tretinoin; 0.1–7.5 μM; Sigma) for 2 h in the presence of 1% FCS and stimulated by H_2O_2 (75–100 μM; Sigma), TNF-α (250 units/ml; a gift from Dr. K. Noguchi, Teikyo University, Japan), or pyrrolidine dithiocarbamate (PDTC; 10–20 μM; Sigma) for up to 24 h. PDTC is supposed to induce apoptosis via activation of pro-apoptotic molecule AP-1 and inactivation of anti-apoptotic molecule NF-κB (31, 32). Compared with mesangial cells, NRK49F cells, MDCK cells, and ECV304 cells were relatively resistant to H_2O_2-induced injury. The following concentrations of H_2O_2 were used for individual cell types: NRK49F, 150–200 μM; MDCK, 400 μM; and ECV304, 200–400 μM. Inhibition of apoptosis with t-RA at concentrations over 7.5–10 μM was generally used for experiments.

Northern Blot Analysis

Expression of c-fos and c-jun was examined by Northern blot analysis (38). In brief, confluent mesangial cells cultured in the presence of 1% FCS were pretreated with t-RA (5 μM) for 2 h and stimulated by 75–100 μM H_2O_2 for 30 min and 2 h. Total RNA was extracted by the single-step method (39) and subjected to analysis. cDNAs for c-Fos (40), c-Jun (41), and glyceraldehyde-3-phosphate dehydrogenase (42) were used as probes.

JNK Assay

Confluent mesangial cells cultured in 6-well plates in the presence of 1% FCS for 24 h were pretreated with t-RA (5 μM) for 2 h and exposed to 100 μM H_2O_2 for 1 h. Activity of JNK was evaluated by phosphorylation of c-Jun, using the SAPK/JNK Assay Kit (New England Biolabs, Herts, United Kingdom) following the protocol provided by the manufacturer.
pressed by the treatment with t-RA (Fig. 1B). Consistently, agarose gel electrophoresis detected DNA ladder formation in H₂O₂-exposed cells, which was markedly attenuated by treatment with t-RA (Fig. 1C).

The apoptotic process is divided into three phases. In the first and second phases, function of cellular membranes is retained intact, but in the third phase, cell membranes are progressively degenerated (33). The final step of apoptosis was, therefore, evaluated by trypan blue exclusion. Confluent mesangial cells were pretreated with or without t-RA and exposed to H₂O₂ (100 μM) for 16 h. After 16 h, both attached and detached cells were gently trypsinized and used for trypan blue analysis. Data are expressed as mean ± S.E. Asterisks indicate statistically significant differences (p < 0.05). Assays were performed in quadruplicate.

The cytotoxic effect of t-RA was dose-dependent. Obvious improvement in cell survival was observed at concentrations higher than 1 μM, and a maximum effect was achieved by 5 μM t-RA (Fig. 1E).

Effect of t-RA on Apoptosis of Mesangial Cells Triggered by Other Stimuli—The anti-apoptotic potential of t-RA was investigated using different stimuli. PDTC is known to induce apoptosis in certain cell types (43–45). The pro-apoptotic action of PDTC is supposed to be via activation of AP-1 and/or inactivation of NF-κB (31, 32). Mesangial cells were pretreated with t-RA and stimulated by PDTC (10–20 μM) in the presence of 1% FCS. Mesangial cells exposed to PDTC showed shrinkage of the cytoplasm. Acridine orange-ethidium bromide staining confirmed that the major mechanism of cell death (82.0 ± 4.4%
after 16 h) was apoptosis. Pretreatment with t-RA reversed the morphologic change (Fig. 2A). Staining of the cells with Hoechst 33258 exhibited condensation and fragmentation of nuclei in PDTC-treated cells. It was suppressed by treatment with t-RA (Fig. 2B, left panel). The relative percentages of apoptotic cells were significantly reduced from 16.8 ± 1.6% (PDTC alone) to 1.0 ± 0.3% (t-RA + PDTC) (versus untreated control, 0.8 ± 0.3%) (Fig. 2B, right panel). Consistently, agarose gel electrophoresis detected DNA fragmentation in PDTC-exposed cells, and it was attenuated by treatment with t-RA (Fig. 2C).

We further tested the effect of t-RA on apoptosis triggered by another apoptosis inducer, TNF-α (46). Like other cell types, cultured mesangial cells are resistant to TNF-α-induced apoptosis. It is due to induction of anti-apoptotic proteins by TNF-α via NF-κB-dependent mechanisms (30, 47). To sensitize mesangial cells to TNF-α-induced apoptosis, we created NF-κB-inactive mesangial cells, SM/IκBαM, by expression of a superrepressor mutant of IκB, IκBαM. The established SM/IκBαM cells exhibited substantial susceptibility to TNF-α-induced cellular injury (30). Acridine orange-ethidium bromide staining confirmed that the major mechanism of cell death (75.8 ± 2.5% after 16 h) was apoptosis. Using the established cells, the effect of t-RA was tested. Microscopic analysis showed that, in contrast to H2O2- and PDTC-initiated apoptosis, t-RA did not affect morphological changes (shrinkage and round-up of the cells) induced by TNF-α (250 units/ml) (Fig. 2D).

FIG. 2. Effect of t-RA on apoptosis of mesangial cells triggered by other stimuli. A, phase-contrast microscopy. Mesangial cells were pretreated with (+) or without (−) t-RA (5 μM) for 2 h in the presence of 1% FCS and exposed to pyrrolidine dithiocarbamate (PDTC; 20 μM) for 24 h. B, Hoechst staining. After the induction of apoptosis (10 μM PDTC), cells were stained by Hoechst 33258. Percentages of condensed and/or fragmented nuclei are shown on the right, mean ± S.E. An asterisk indicates a statistically significant difference (p < 0.05). C, ladder detection assay. Mesangial cells were pretreated with or without t-RA for 2 h, exposed to PDTC (10 μM) for 24 h, and subjected to agarose gel electrophoresis. D, phase-contrast microscopy. Nuclear factor-κB-inactive mesangial cells, SM/IκBαM, were pretreated with (+) or without (−) t-RA (5 μM) for 2 h in the presence of 1% FCS and exposed to TNF-α (250 units/ml) or H2O2 (100 μM) for 24 h. E, Hoechst staining. After the induction of apoptosis, SM/IκBαM cells were stained by Hoechst 33258 and examined by fluorescence microscopy. F, ladder detection assay. SM/IκBαM cells were pretreated with or without t-RA for 2 h, exposed to TNF-α or H2O2 for 24 h, and subjected to agarose gel electrophoresis.

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duced by TNF-α was unaffected by the pretreatment with t-RA (Fig. 2F). In contrast, DNA laddering induced by H₂O₂ was inhibited by t-RA in SM/IκBαM cells.

Effect of t-RA on Apoptosis in Other Cell Types Triggered by H₂O₂—To examine whether the anti-apoptotic effect of t-RA against H₂O₂ is specific to mesangial cells, NRK49F fibroblasts, MDCK epithelial cells, and ECV304 endothelial cells were tested. Dose-dependent effects of H₂O₂ on individual cell type was initially examined to determine minimum concentrations required for cellular damage. Compared with mesangial cells, NRK49F, MDCK, and ECV304 cells were found to be relatively resistant to H₂O₂-induced injury. The minimum concentrations required were 150–200 μM for NRK49F cells, 400 μM for MDCK cells, and 200–400 μM for ECV304 cells (data not shown). Using these concentrations, effects of t-RA on H₂O₂-induced apoptosis were examined.

NRK49F fibroblasts exposed to H₂O₂ exhibited shrinkage of the cytoplasm, membrane blebbing, and condensation of nuclei. Pretreatment with t-RA substantially inhibited these morphologic changes (Fig. 3A). Hoechst staining showed condensation and fragmentation of nuclei in H₂O₂-exposed cells, whereas it was suppressed by treatment with t-RA (Fig. 3B, left panel). The percentages of apoptotic cells were significantly reduced from 35.0 ± 5.9% (H₂O₂ alone) to 15.0 ± 1.7% (t-RA + H₂O₂) (versus untreated control, 3.7 ± 1.1%) (Fig. 3B, right panel). Consistently, agarose gel electrophoresis detected DNA ladder formation in H₂O₂-exposed NRK49F cells, and it was attenuated by treatment with t-RA (Fig. 3C).

In contrast to mesangial cells and NRK49F fibroblasts, t-RA did not diminish H₂O₂-induced apoptosis of MDCK cells. Morphological analysis, Hoechst staining, and agarose gel electrophoresis showed typical features of apoptosis in H₂O₂-exposed

**Fig. 3.** Effect of t-RA on apoptosis of other cell types triggered by H₂O₂. NRK49F fibroblasts and MDCK epithelial cells were pretreated with (+) or without (−) t-RA (5 μM) for 2 h in the presence of 1% FCS and exposed to H₂O₂ (150–200 μM for NRK49F cells, 400 μM for MDCK cells) for 24 h. Cells were then subjected to phase-contrast microscopy (A and D), Hoechst staining (B and E) and agarose gel electrophoresis (C and F). An asterisk indicates a statistically significant difference (p < 0.05).
MDCK cells, and the apoptotic process was not affected by the pretreatment with t-RA (Fig. 3, D-F). The percentages of apoptotic cells were 17.0 ± 0.8% in H₂O₂ alone and 14.7 ± 1.0% in t-RA + H₂O₂ (Fig. 3E, right panel, not statistically different). Similar unresponsiveness to t-RA was observed in ECV304 endothelial cells (data not shown).

Effect of t-RA on the JNK-AP-1 Pathway—Activation of AP-1 is a crucial signaling event that mediates H₂O₂-induced apoptosis in mesangial cells (10, 11). We examined the effect of t-RA on the activity of AP-1, especially focusing on expression of AP-1 components and activation of JNK. In the presence of serum (10%), mesangial cells exhibit constitutive AP-1 activity. Reporter assays showed that t-RA (5 μM) significantly suppressed the basal activity of AP-1 (Fig. 4A). Compared with the untreated control (100 ± 9.5%), the activity of AP-1 was decreased to 48.2 ± 5.4% by the treatment with t-RA.

The effect of t-RA on the oxidant-induced activation of AP-1 was further examined by reporter assays. In response to H₂O₂, mesangial cells exhibited up-regulation of AP-1 activity (196.4 ± 21.7%). Pretreatment with t-RA abrogated the H₂O₂-induced activation of AP-1 (106.0 ± 7.2%) (Fig. 4B).

To identify molecular mechanisms involved in the suppressive action of t-RA on AP-1, its effect on the expression of c-fos and c-jun was examined. Mesangial cells were pretreated with or without t-RA for 2 h and stimulated by H₂O₂ for 0.5 and 2 h. Northern blot analysis detected substantial induction of c-fos and c-jun mRNAs in response to H₂O₂. Pretreatment with t-RA completely abolished the oxidant-induced expression of c-fos and c-jun (Fig. 4C).

The activity of AP-1 is regulated by phosphorylation-dependent activation by JNK. We therefore examined whether or not t-RA affects the activity of JNK. Mesangial cells were pretreated with or without t-RA, stimulated by H₂O₂ for 1 h and subjected to the JNK assay. After stimulation with H₂O₂, substantial induction of JNK activity was observed. Pretreatment with t-RA markedly diminished the activation of JNK in response to H₂O₂ (Fig. 4D).

To examine whether JNK is required for the H₂O₂-induced apoptotic, mesangial cells were transiently co-transfected with an empty plasmid or an expression plasmid encoding a dominant-negative mutant of JNK1 together with pCI-βGal that introduces a β-galactosidase gene. After incubation for 24 h in the presence of 1% FCS, cells were treated with H₂O₂ for 12 h and subjected to X-gal assay. Percentages of shrunk/rounded blue cells (apoptotic cells) against total numbers of blue cells were evaluated. As shown in Fig. 4E, treatment with H₂O₂ significantly increased round cells in mock-transfected cells (2.4 ± 0.2 fold versus untreated control, p < 0.05). In contrast, in the cells transfected with the JNK mutant, significant increase of apoptotic cells was not observed after exposure to H₂O₂ (1.1 ± 0.1-fold versus untreated control).

**DISCUSSION**

RA has been considered as a potential therapeutic agent for malignant diseases, especially for the treatment of leukemia (25). It is based on the pharmacological potential of RA to induce growth arrest, cellular differentiation, and apoptosis (48). RA triggers apoptosis of a variety of cell types including embryonic cells and tumor cells. In contrast, little is known about anti-apoptotic action of RA. Previous studies have shown that RA may inhibit apoptosis of T cells, leukemic cells, and hematopoietic cells (49–52). Currently, it is unknown whether RA inhibits apoptosis of non-leukocyte lineage. Using H₂O₂ as a trigger, the present report provides novel evidence for the anti-apoptotic potential of RA. Our data showed that t-RA attenuates H₂O₂-induced apoptosis in mesangial cells and fibroblasts. The molecular mechanisms involved in its anti-apoptotic action was not fully elucidated, but the current results suggested that the JNK-AP-1 pathway is one of its potential targets. The fact that t-RA inhibited apoptosis triggered by
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PDTC, another activator of AP-1 (31), further supported this possibility.

RA has been generally regarded as an inhibitor of AP-1 (23). However, previous studies indicated that the manner of which RA affects the AP-1 pathway varies from cell type to cell type. For example, RA inhibits expression of c-fos and c-jun in synovial fibroblasts (53). In human bronchial epithelial cells, growth-factor-induced activation of JNK is also inhibited by RA (54). However, in vascular smooth muscle cells, RA inhibits AP-1 activity without suppressing expression of c-fos and c-jun (55). In human skin, RA inhibits ultraviolet-triggered accumulation of c-Jun via a post-transcriptional mechanism (56). RA inhibits AP-1 activity without suppressing expression of c-fos and c-jun (55).

Interestingly, we found that TNF-α-induced apoptosis was not inhibited by t-RA. This result may lead to some confusion, because (i) TNF-α induces apoptosis via generation of ROI (73–75), and (ii) t-RA suppresses ROI-induced apoptosis, as shown in this report. A possible explanation for this is that ROI other than H_2O_2, e.g. superoxide anion (O_2^-), may be involved in the TNF-α-induced apoptosis and that t-RA selectively inhibits the action of H_2O_2, but not other ROI. Our recent data support this possibility. That is, we found that scavengers of O_2^- but not scavengers of H_2O_2 inhibited TNF-α-induced apoptosis in mesangial cells. Furthermore, in contrast to H_2O_2-triggered apoptosis, apoptosis induced by O_2^- releasing agents was not inhibited by t-RA. Taken together, these data support the idea that t-RA suppresses the action of particular ROI including H_2O_2.

The reason for the lack of effects of t-RA on MDCK cells and ECV304 cells is unknown. As described above, the anti-AP-1 action of t-RA is different from cell type to cell type. The different responses to t-RA may be due to different effects of t-RA on the JNK-AP-1 pathway in different cell types. Alternatively, different levels of expression of RARs and RXRs might have caused the different responsiveness to t-RA. Further investigation is required to examine these possibilities.

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