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Role of Retinoic Acid in Spontaneous Apoptosis of Human Neutrophils

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Although retinoic acid has been known as either anti-inflammatory or pro-inflammatory molecule, depending on the cell type, its exact role in mature human neutrophils has not been fully explored. In this study, we investigate the effects of retinoic acid on neutrophil apoptosis and the associated mechanism and found that 9-cis retinoic acid (9CRA) significantly inhibits the spontaneous apoptosis of neutrophils. Its effect is increased by co-treatment with TNF-α ($P<0.05$). The 9CRA-induced inhibition is blocked by the following enzyme inhibitors: Ly 294002, phosphoinoside (PI)-3 kinase inhibitor, U73122, a phospholipase C (PLC) inhibitor, PP2, Src family protein inhibitor, SB202190, p38 MAPK inhibitor, and BAY-11-7085, NF-κB inhibitor. This study also demonstrates that all-trans retinoic acid suppresses spontaneous apoptosis, similar to the mechanism of inhibition exhibited by 9CRA. Phosphorylation of p38 MAPK decreases by 9CRA treatment. Iκ-Bα is degraded until 30 minutes after a time-dependent 9CRA treatment, but degradation can be inhibited by Ly 294002. These results indicate that 9CRA decreases p38 MAPK activation, induces NF-κB activation via PI-3 kinase, and also blocks cleavage of caspase 3. As these findings suggest, 9CRA has a molecular mechanism which may help pro-inflammatory response by blocking neutrophil apoptosis.

Key Words: Retinoic acid, Neutrophils, Apoptosis

INTRODUCTION

Neutrophils in peripheral blood are polymorphonuclear leukocytes that have primary and specific granules. They have a short life span. In early immune response, neutrophils move from the blood into the infected tissues and then die soon after removing the pathogen. Neutrophils are killed via spontaneous apoptosis. Neutrophil apoptosis includes both intrinsic and extrinsic pathway. The intrinsic route is believed to be a main apoptotic process in tissues (Webb et al., 2000; Maianski et al., 2004). A variety of extracellular stimulators modulate neutrophil death by inhibiting spontaneous apoptosis (Simon, 2003). In some inflammatory diseases, including respiratory distress syndrome and rheumatoid arthritis, symptoms are caused by abnormal or decreased neutrophil apoptosis (Webb et al., 2000; Maianski et al., 2004).

Retinoic acid (RA) as a lipophilic molecule is derived from vitamin A. It is classified into three stereoisomers: 9-cis retinoic acid (9CRA), all-trans retinoic acid (ATRA), and 13-cis retinoic acid. The major receptors binding to RA include the retinoid X receptor (RXR) and the retinoic acid receptor (RAR) (Evans, 1988; Pemrick et al., 1994). RA is believed to play vital roles on immune response by modulating growth, differentiation, apoptosis and chemotaxis of leukocytes (Stephensen, 2001). As has been recently reported, RA supports an inflammatory process by increasing expression of the pro-inflammatory cytokine, IL-1β and TNF-α (Hayashi et al., 1996; Landis et al., 2002). In contrast to the pro-inflammatory effects, RA also provokes anti-inflammatory effects (Gille et al., 1997; Kreutzet al., 1998; Na et al., 1998).

In this study, we investigated on whether RA has pro-inflammatory or anti-inflammatory effect on neutrophil

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apoptosis. We furthermore examined the mechanism on how it regulates neutrophil apoptosis.

MATERIALS AND METHODS

1. Materials

RPMI 1640 and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. (Gaithersburg, MD). 9CRA and ATRA were obtained from Sigma (St. Louis, MO). Ly 294002, U73122, rottlerin, Ro-31-8425, PD98059, PP2, SB202190, AG490 and BAY-11-7085 were obtained from Calbiochem (San Diego, CA). TNF-α was obtained from R&D Systems (Minneapolis, MN). Anti-phospho-p38 MAPK, anti-p38 MAPK, anti-Iκ-Bα, anti-ERK2, anti-caspase 3 and anti-caspase 9 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2. Isolation of human neutrophils

Human neutrophils were isolated from heparinized peripheral blood of healthy volunteers in Eulji University by

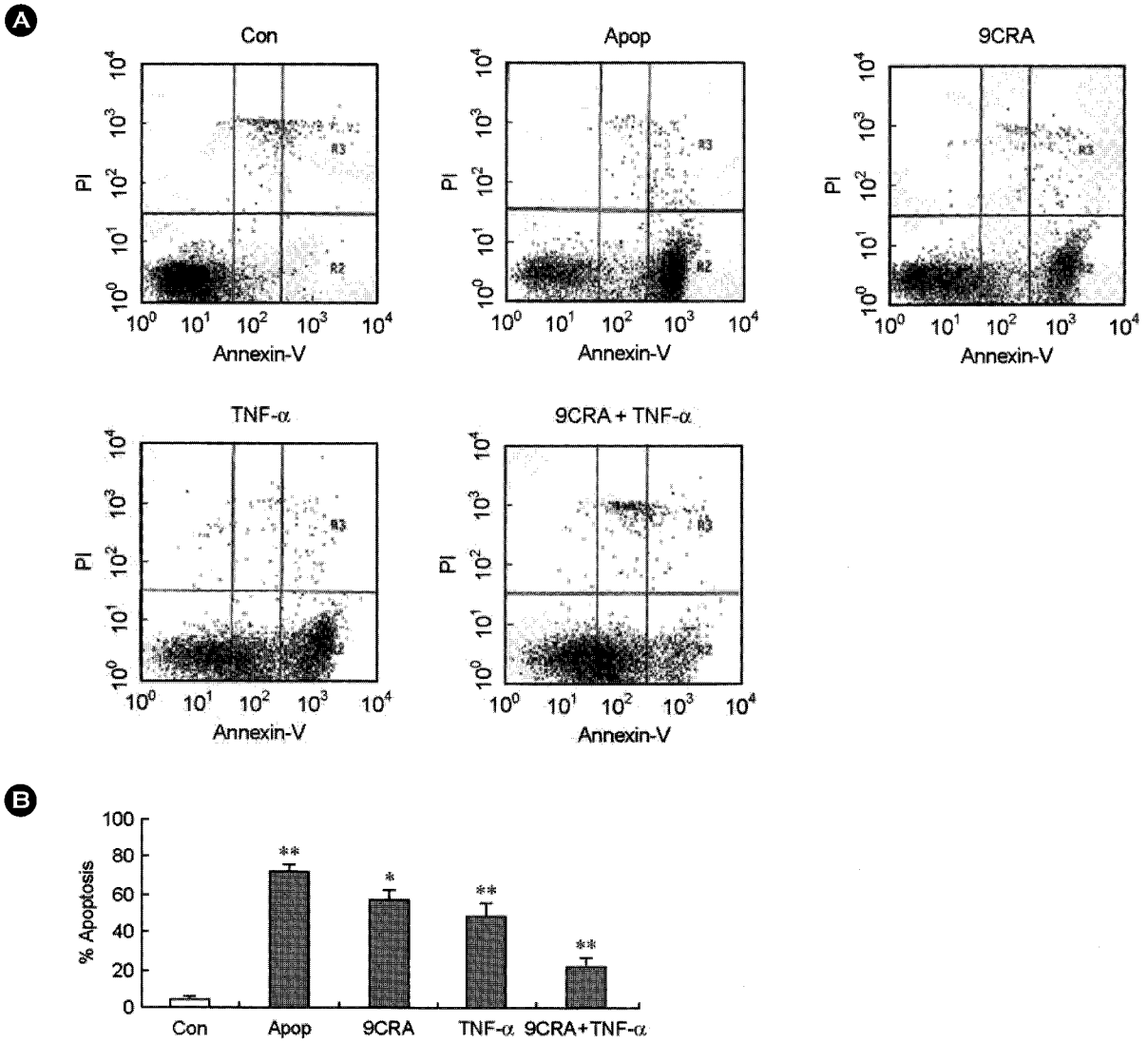

Fig. 1. 9CRA inhibits the spontaneous apoptosis of human neutrophils. **A,** Human neutrophils were isolated in peripheral blood by Ficoll-Hypaque solution. After treatment without (Apop) or with 1 μM 9CRA, 10 ng/ml TNF-α or 1 μM 9CRA and 10 ng/ml TNF-α for 24 h, the cells were harvested and resuspended in binding buffer. The cells were incubated with annexin V-FITC and PI and analyzed by FACSort cytofluorimeter using CELLQUEST software. Fresh neutrophils were considered as negative control (Con). Apoptotic cells were evaluated as a percentage of annexin V-positive cells among total cells. Data are expressed as representatives of ten individual experiments. **B,** Data are presented as the means ± S.E.M. in ten individual experiments and $^{*}P<0.05$ and $^{**}P<0.01$ were accepted as a significant difference between the normal (Con) group and untreated (Apop) group or between the untreated group and stimulator-treated group.

Ficoll-Hypaque solution (Amersham Pharmacia Biotechnology, Buckinghamshire, U.K.). After removal of erythrocytes using RBC lysis solution (Sigma), the neutrophils were washed three times by PBS and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 g/ml). The cells were seeded at a concentration of 3×10^6 cells/ml in 24 well plates. Cell viability was at least 90% as evaluated by the trypan blue exclusion test. Purity of neutrophils was above 97% as assessed under a microscope after Wright staining of cytocentrifuged samples.

3. Detection of neutrophil apoptosis

AnnexinV-fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, and Company Franklin Lakes, NJ) was used for measuring apoptotic cells. After treatment with 9CRA, ATRA or TNF-α, cells were harvested and resuspended in binding buffer at a concentration of 5×10^4 cells. Annexin V-FITC and PI were added and incubated for 15 min at room temperature. The cells were analyzed by FACSort cytofluorimeter using CELLQUEST software. Apoptotic cells were evaluated as a percentage of annexin-positive cells among total cells.

4. Western blotting

Cell extracts were prepared by homogenization in lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). Protein samples (50 g/well) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated with primary antibodies against a target protein and thereafter incubated with secondary antibodies against primary antibodies. Finally, the membranes were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). The stripped membranes were reprobed with anti-ERK2 or anti-p38 antibodies for normalization.

5. Statistical analysis

Data are expressed as the means ± S.E.M. Statistical differences were analyzed using one-way ANOVA. The SPSS statistical software package (Version 10.0, Chicago, IL) was used for statistical analysis. Asignificant P value was defined as less than 0.05.

RESULTS

1. 9CRA inhibits the spontaneous apoptosis of human neutrophils

We first performed annexin V and PI stain for examining whether 9CRA affectspontaneous neutrophil apoptosis. 9CRA decreased apoptotic cells to 56.4%, compared with untreated neutrophils (72.6%) (Fig. 1). TNF-α is known as an anti-apoptotic molecule. We next co-treated neturophils with 9CRA and TNF-α to confirm the inhibitory effect of 9CRA and to examine its synergistic effect with TNF-α.

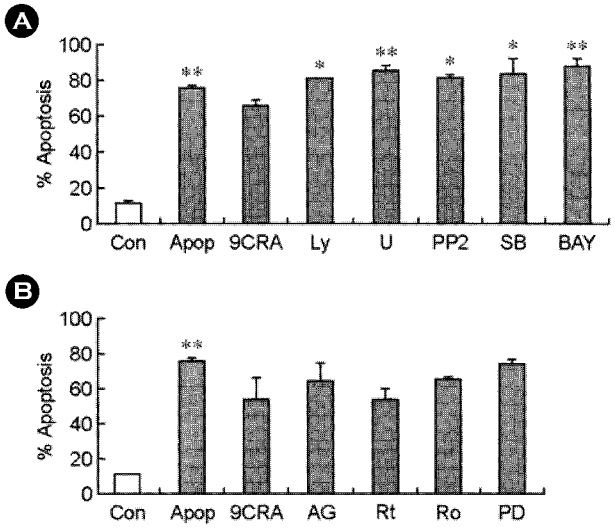

Fig. 2. Inhibition of neutrophil apoptosis due to 9CRA is associated with PI-3 kinase, PLC, Src family protein kinase, p38 MAPK and NF-κB. **A,** Isolated neutrophils were pretreated in the absence or the presence of 10 μM Ly 294002 (Ly), 1 μM U73122 (U), 10 μM PD98059 (PD), 10 μM SB202190 (SB), 10 μM PP2 or 10 μM BAY-11-7085 (BAY) for 30 min. **B,** Isolated neutrophils were pretreated in the absence or the presence of 10 μM AG490 (AG), 5 μM rottlrerin (Rt), 100 μM Ro-31-8425 (Ro), 10 μM PD-98059 (PD) for 30 min. After the treatment without (Apop) or with 1 μM 9CRA for 24 h, the cells were harvested and resuspended in binding buffer. The cells were incubated with annexin V-FITC and PI and analyzed by FACSort cytofluorimeter using CELLQUEST software. Fresh neutrophils were considered as negative control (Con). Apoptotic cells were evaluated as a percentage of annexin V-positive cells among total cells. Data are presented as the means ± S.E.M. in ten individual experiments and $^*P<0.05$ and $^{**}P<0.01$ were accepted as a significant difference between the normal (Con) group and untreated (Apop) group, between the untreated group and 9CRA-treated groupor between the 9CRA-treated group and inhibitor-treated group.

Both 9CRA and TNF-treatment show more effective suppression than single stimulation by 9CRA.

2. Inhibition of neutrophil apoptosis due to 9CRA is associated with PI-3 kinase, PLC, Src family protein kinase, p38 MAPK and NF-κB

To further understand how 9CRA blocks neutrophil apoptosis, we examined the alternation of the effect by using specific inhibitors against signaling proteins. As shown in Fig. 2A, spontaneous apoptosis was significantly blocked by Ly 294002, U73122, PP2, SB202190 and BAY-11-7085 ($P<0.05$). However, other inhibitors such as AG490, rottlerin, Ro-31-8425, and PD98059 have no effect on the spontaneous apoptosis (Fig. 2B). Since retinoids can show similar or different immunological responses according to their isoforms, we examined whether ATRA has a different effect on neutrophil apoptosis, compared with that of 9CRA (Babina et al., 2001; Koistinen et al., 2002; Huang et al., 1988; Sakashita et al., 1993). ATRA acted as an inhibitor against neutrophil apoptosis similar to role of 9CRA (Fig. 3). In experiments using signaling protein inhibitors, ATRA-induced anti-apoptotic mechanism was almost similar to 9CRA-induced mechanism, despite the differences in the degree of inhibition.

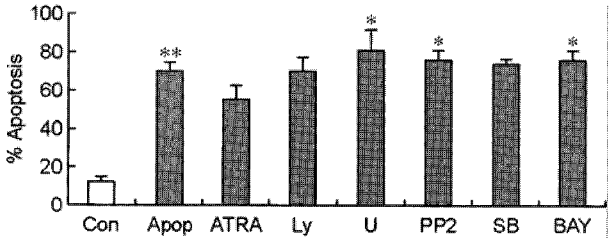

Fig. 3. ATRA inhibits neutrophil apoptosis similar to 9CRA-induced mechanism. Isolated neutrophils were pretreated in the absence or the presence of 10 μM Ly 294002 (Ly), 1 μM U73122 (U), 10 μM PP2, 10 μM SB202190 (SB) or 10 μM BAY-11-7085 (BAY) for 30 min. After the treatment without (Apop) or with 1 μM ATRA for 24 h, the cells were harvested and resuspended in binding buffer. The cells were incubated with annexin V-FITC and PI and analyzed by FACSort cytofluorimeter using CELLQUEST software. Fresh neutrophils were considered as negative control (Con). Apoptotic cells were evaluated as a percentage of annexin V-positive cells among total cells. Data are presented as the means ± S.E.M. in five individual experiments and $^{*}P<0.05$ and $^{**}P<0.01$ were accepted as a significant difference between the normal (Con) group and untreated (Apop) group, between the untreated group and ATRA-treated group or between the ATRA-treated group and inhibitor-treated group.

3. RA inhibits the phosphorylation of p38 MAPK and increases Iκ-Bα degradation

Since SB202190 and BAY-11-7085 block the inhibition of apoptosis due to 9CRA, we examined alternation of p38 MAPK and Iκ-Bα after the treatment with 9CRA by performing Western blotting studies. Phosphorylation of p38 MAPK begins to decrease at 5 min after spontaneous apo-

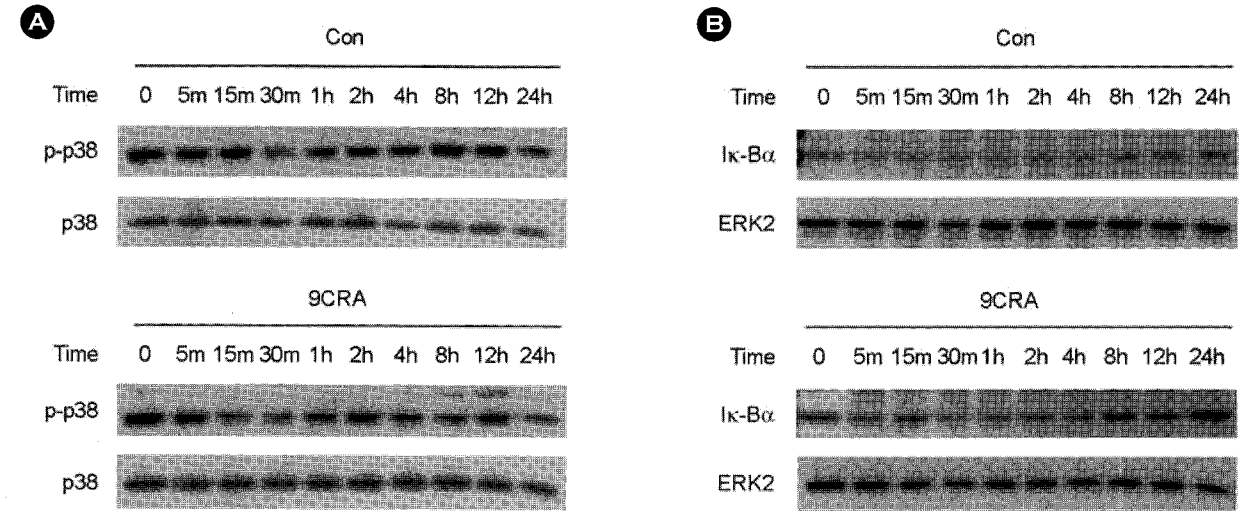

Fig. 4. RA inhibits the phosphorylation of p38 MAPK and increases Iκ-Bα degradation. Isolated neutrophils were incubated without or with 1 μM 9CRA for the indicated time. The harvested cells were lysed and the extracted proteins were separated on 10% SDS-polyacrylamide gels (50 μg/lane) and transferred to nitrocellulose membrane. Phosphorylation of p38 MAPK **(A)** and degradation of Iκ-Bα **(B)** were detected by Western blotting with anti-phospho p38 MAPK and anti-Iκ-Bα antibodies, respectively. The membrane was stripped and reprobed with p38 MAPK and ERK2 antibodies as an internal control.

ptosis. This decrease in phosphorylation peaked at 30 min without the alternation of p38 MAPK expression. 9CRA inhibited more strongly than the level of phospho-p38 MAPK at 15 min and 30 min (Fig. 4A). Iκ-Bα expression after exposure of neutrophils to 9CRA showed stronger inhibition than the control at a 30 min, a time of peak inhibition. The decrease was recovered degree of inhibition decreased after the peak time, but it was sustained for up to 24 h (Fig. 4B). These results indicate that 9CRA regulates neutrophil apoptosis by modulation of p38 MAPK activation and Iκ-Bα expression related with NF-κB activation.

4. Degradation of Iκ-Bα due to 9CRA is involved in PI-3 kinase

We next examined the upstream molecules of p38 MAPK and Iκ-Bα by using signal protein-specific inhibitor. 9CRA decreased phosphorylated p38 MAPK expression, in agreement with the results shown in Fig. 5A, but U73122, PP2 and Ly 294002 have no effect on the expression. Iκ-Bα degradation was reversed by Ly 294002 and the decrease was not affected by both U73122 and PP2 (Fig. 5B).

5. 9CRA inhibits caspase 3 activation

As shown in Fig. 6, caspase 3 decreased in a time-dependent manner, indicating increased cleavage formation or activation of caspase 3. 9CRA blocked cleavage of caspase 3 at 12 h and 24 h. These results suggest that 9CRA inhibits neutrophil apoptosis by blocking caspase 3 activation.

DISCUSSION

Retinoic acidacts as an essential modulator in immune responses including leukocyte recruitment and activation. Although RA has been known to induce differentiation and apoptosis of myeloid cells, its function on mature neutro-

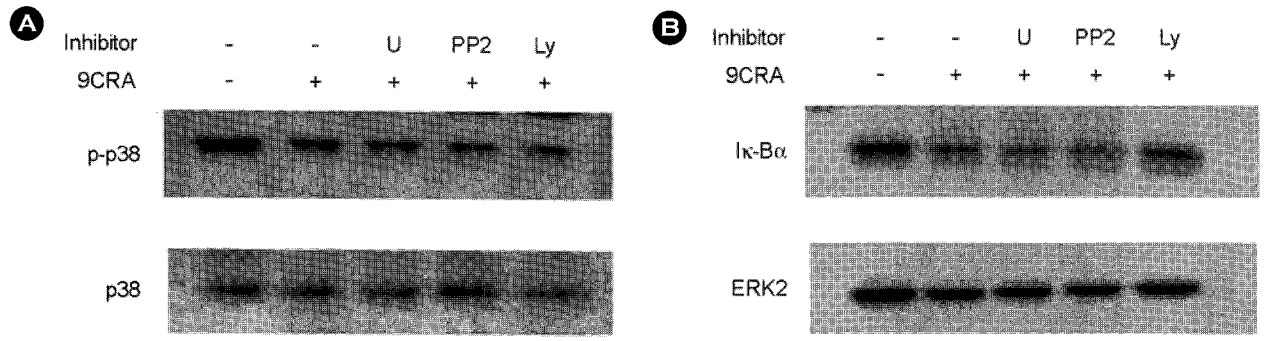

Fig. 5. Degradation of Iκ-Bα due to 9CRA is involved in PI-3 kinase. Isolated neutrophils were pretreated in the absence or the presence of 1 μM U73122 (U), 10 μM PP2 or Ly 294002 (Ly) for 30 min. After the treatment without or with 1 μM 9CRA for 24 h, the harvested cells were lysed and the extracted proteins were separated on 10% SDS-polyacrylamide gels (50 μg/lane) and transferred to nitrocellulose membrane. Phosphorylation of p38 MAPK **(A)** and degradation of Iκ-Bα **(B)** weredetected by Western blotting with anti-phospho p38 MAPK and anti-Iκ-Bα antibodies, respectively. The membrane was stripped and reprobed with p38 MAPK and ERK2 antibodies as an internal control.

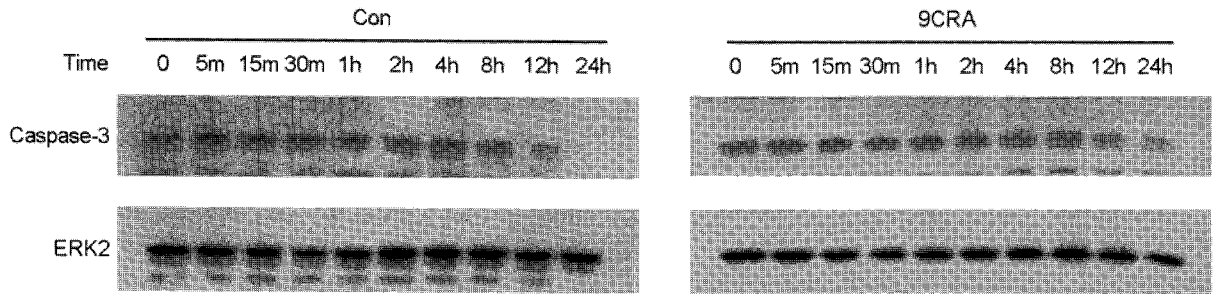

Fig. 6. 9CRA inhibits activation of caspase 3. Isolated neutrophils were incubated without (Con) or with 1 μM 9CRA for the indicated time. Harvested cells were lysed and extracted proteins were separated on 10% SDS-polyacrylamide gels (50 μg/lane) and transferred to nitrocellulose membrane. Casepase 3 expression wasdetected by Western blotting with anti-caspase 3 antibodies. The membrane was stripped and reprobed with ERK2 antibodies as an internal control.

phils in peripheral blood has not been precisely understood. In this study, we investigated the effects of RA on neutrophil apoptosis and demonstrated that (1) 9CRA blocks spontaneous apoptosis (2) anti-apoptotic mechanism due to 9CRA involves p38 MAPK and NF-κB; (3) ATRA inhibits apoptosis through a mechanism similar to that of 9CRA and (4) 9CRA blocks the activation of caspase 3.

RA induces maturation of myeloid cells into segmented neutrophils and the successive treatment activates apoptosis, following cell death. As shown in Fig. 1, 9CRA blocked the apoptosis of mature neutrophils. The anti-apoptotic mechanism is involved in signaling proteins such as PI-3 kinase, PLC, Src family protein, p38 MAPK and NF-κB (Fig. 2). In the regulation of p38 MAPK by 9CRA, we found unexpectedly that 9CRA decreases p38 MAPK activation (Fig. 4 and 5). The basal level of p38 MAPK activation is high and it decreased gradually during neutrophil apoptosis. Although it has been controversially reported that p38 MAPK is an apoptotic molecule or an anti-apoptotic protein, p38 MAPK may be considered as apoptotic molecule in this experiment (Aoshiba et al., 1999; Avarado-Kristensson et al., 2004). The decrease of phosphorylated p38 MAPK may indicate a survival signal for blocking a death signal after beginning apoptosis through a still unknown mechanism, and 9CRA induces neutrophil apoptosis by decreasing p38 MAPK activation, in agreement with the result of complete inhibition of neutrophil apoptosis after the treatement with 9CRA plus SB202190 (Fig. 2A). In addition, 9CRA induces NF-κB activation via PI-3 kinase by Iκ-Bα degradation and this mechanism is similar to the survival mechanism induced by IFN-γ (Fig. 5) (Simon, 2003).

Since RAR and RXR binding to RA are nuclear receptors, the molecular complex of RA and its receptor operate as a transcription factor and results in enhancing transcription and translation. Recently, several reports demonstrated that RA indirectly or directly activates signaling proteins existing in cytosol (Bost et al., 2002; Antonyak et al., 2003 Ko et al., 2007). Our results showed that RA induces dephosphorylation of p38 MAPK and activates NF-κB by Iκ-Bα degradation (Fig. 4), although the activation mechanism induced by RA remains to be determined. Neutrophil apoptosis consists of both extrinsic and intrinsic pathway. Extrinsic pathway begins after interaction of extracellular ligands with their receptors (Simon, 2003). The intrinsic pathway is induced by mitochondrial stress, following the alternation of Bcl-2 family proteins. Caspase 3 is a major protein in both the intrinsic and extrinsic process. 9CRA blocks neutrophil apoptosis by inhibition of caspase 3 as anti-apoptosis signal as well as NF-κB activation as a survival signal (Fig. 6).

Role of RA on inflammation has remained controversial. It has been recently reported that RA increases adhesion molecules and chemokine receptor as well as cytokine release (Babina et al., 2001; Babina and Henz, 2003; Kim et al., 2004; Ko et al., 2006; Lee et al., 2007). In comparison with the pro-inflammatory effect, RA blocks the expression of adhesion molecules in endothelial cells and cytokine production in macrophages (Gille et al., 1997; Na et al., 1999). Our data indicate that RA has a possible pro-inflammatory function by inhibiting neutrophil apoptosis. The conflicting results about the effects of RA in immune response indicate that RA may have different effects in response to a cell type, and inflammation modulated by RA also may exist as a different phenotype under a variety of circumstances.

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