Retinoic Acids Are Potent Inhibitors of Spontaneous Human Eosinophil Apoptosis

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Retinoic acids (RAs), which are active metabolites of vitamin A, are known to enhance Th2-type immune responses in vitro, but the role of RAs in allergic inflammatory cells remains unclear. In this study, we demonstrated that purified peripheral blood eosinophils expressed nuclear receptors for RAs at the mRNA and protein levels. Eosinophils cultured with all-trans RA (ATRA) and 9-cis-RA showed dramatically induced cell survival and nuclear hypersegmentation, and the efficacy of RAs (10−9M) was similar to that of IL-5 (1 ng/ml), the most critical cytokine for eosinophil activation. Pharmacological manipulation with receptor-specific agonists and antagonists indicated that the antipapoptotic effect of RAs was mediated through ligand-dependent activation of both retinoid acid receptors and retinoid X receptors (mainly retinoid acid receptors). Furthermore, using a gene microarray and a cytokine Ab array, we discovered that RAs induced vascular endothelial growth factor, M-CSF, and MCP-1 secretion, although they were not involved in eosinophil survival. RA-induced eosinophil survival appears to be associated with down-regulation of caspase 3 and inhibition of its enzymatic activity. These findings indicate an important role of RAs in homeostasis of granulocytes and provide further insight into the cellular and molecular pathogenesis of allergic reactions. The Journal of Immunology, 2008, 181: 7689–7698.

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Th1 and enhance Th2 development (16). Despite these results that suggest that vitamin A biases the response in a Th2 direction, the influence of RA on allergic disease is limited and appears contradictory. For instance, supplementation with a natural source of vitamin A had a protective effect against exercise-induced asthma in some patients (17). Several epidemiological studies suggested that vitamin A intake had no association with or protective effect on asthma (18). In contrast, RA can induce eosinophilia and exacerbation of asthma under certain conditions (19). Therefore, it is of paramount importance to clarify the regulation of immune responses by RA and its underlying cellular and molecular mechanisms.

The direct effect of RA on allergic inflammatory cells, especially eosinophils, is less well understood. In the present study, we investigated the functional roles of RA on purified human eosinophils. We found that peripheral blood eosinophils expressed receptors for RAs and, interestingly, ATRA and 9-cis-RA dramatically inhibited spontaneous eosinophil apoptosis. The effect exerted by RA was parallel with the down-regulation of caspase 3 transcription and its enzymatic activity. Together, our experiments showed that RAs activated eosinophils to produce several proinflammatory cytokines.

**Materials and Methods**

**Materials**

9-cis-RA and ATRA were from Sigma-Aldrich. The synthetic RAR agonist [(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) and the synthetic RARα antagonist Ro-41-5253 were purchased from BioMol Research Laboratories. RXR agonist HX630 was a gift from Dr. H. Kagechika (University of Tokyo, Japan) (20). All retinoids were dissolved in DMSO (Sigma-Aldrich) at 5–25 mM stock solutions, protected from light, and stored at −70°C.

**Cell preparation**

Peripheral venous blood was obtained from subjects with mild eosinophilia. Informed consent was obtained from each subject, and the study protocol was approved by the Ethics Committee of Akita University School of Medicine. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia) density gradients (21, 22). The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec). The eosinophils (>98% purity) were then suspended in HBSS (open histograms) and receptor expression (filled histograms). Results are representative of n > 3.

**RT-PCR**

Total RNA was extracted with the use of Ultraspec RNA (Biotex Laboratories) from eosinophils. The total RNA was reverse transcribed with 3 μg of RNA using an Omniscript reverse transcriptase (RT) kit (Qiagen) according to the manufacturer’s protocol. One microliter of the cDNA solutions, protected from light, and stored at −20°C. All retinoids were dissolved in DMSO (Sigma-Aldrich) at 5–25 mM stock solutions, protected from light, and stored at −70°C.

**Immunoblotting**

Eosinophils or monocytes (2 × 10⁶ cells) were lysed in a lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na₂VO₄, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol, 1 mg/ml aprotinin, leupeptin, and pepstatin). After 20 min on ice, detergent-insoluble materials were removed by centrifugation at 4°C at 12,000 × g. The supernatants were mixed with SDS sample buffer and boiled for 4 min. SDS-polyacrylamide (10%) gels (Ready Gel J) were obtained from Bio-Rad. The electrophoresed gel was blotted onto Hybond ECL membranes (Amersham). Excess binding sites were blocked by incubation with 10% BSA in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20, pH 7.6) for 1 h followed by incubation in the primary Ab overnight at 4°C (rabbit polyclonal anti-RARα, β, or γ and RXRα, β, or γ Abs) from Santa Cruz Biotechnology. After washing three times with TBS-T, the membrane was incubated with the secondary Ab (0.04 mg/ml HRP-conjugated goat anti-rabbit IgG Ab from Santa Cruz Biotechnology) for 30 min. The blots were visualized by an ECL system (Amersham) according to the manufacturer’s instructions.

**FIGURE 1.** The expression of RARs and RXRs in human eosinophils. A, The mRNA expression profile was compared with freshly isolated human monocytes using RT-PCR. B, Western blot analysis for protein expression of RARs and RXRs in eosinophils from two different donors. C, Flow cytometric analysis confirming the findings of Western blotting. Purified human peripheral blood eosinophils were permeabilized and then stained with indicated Abs. Histograms show the isotype-matched control (open histograms) and receptor expression (filled histograms). Results are representative of n > 3.

**TABLE 1.** The mRNA expression profile was compared with freshly isolated human monocytes using RT-PCR.
Flow cytometric analysis for retinoid receptors

Intercellular staining was performed to detect nuclear retinoid receptors in eosinophils using a Fix and Perm Cell Permeabilization Kit (Caltag Laboratories). The purified eosinophils were washed twice with PBS and then stained with 1/500 diluted rabbit polyclonal anti-RARα/H9251, /H9252, or /H9253 and RXRα/H9251 or /H9252 Abs (Santa Cruz Biotechnology) and PE-conjugated, affinity-purified anti-rabbit IgG (Rockland). Rabbit IgG Ab (DakoCytomation) was used as an isotype-matched control. The stained cells were analyzed using a flow cytometer (FACScan; BD Immunocytometry Systems).

Cell culture

Purified eosinophils were resuspended at 0.5 × 10⁶ cells/ml in RPMI 1640 medium (Life Technologies) with 10% FCS and incubated with or without indicated concentrations of 9-cis-RA, ATRA, TTNPB, HX630, 0.1% DMSO (vehicle), IL-5 (Genzyme), or GM-CSF (Genzyme) at 37°C in humidified air with 5% CO₂ for 4–72 h. In some experiments, eosinophils were preincubated with or without neutralizing anti-IL-5 or anti-GM-CSF Ab (Genzyme). The RPMI 1640 was supplemented with 2 mM l-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Eosinophils were then examined for each analysis. We confirmed that the effect of DMSO was negligible at the concentration used in each experiment.

Determination of cell death and apoptosis by flow cytometry and morphological analysis

An apoptosis detection kit (Medial Biological Laboratories) was used to quantitatively determine eosinophils undergoing apoptosis by virtue of their ability to bind to annexin V and propidium iodide (PI). Briefly, harvested eosinophils were washed twice in cold PBS and stained with annexin V and PI according to the manufacturer’s instructions. Eosinophil apoptosis and viability were analyzed using a BD Biosciences FACScan cytometer (22). To evaluate apoptosis and nuclear hypersegmentation, cytospins of eosinophils recovered from different cultures were prepared and

FIGURE 2. The effect of RAs on eosinophil survival. A, After incubation with IL-5 (1 ng/ml), 9-cis-RA (10⁻⁶ M), or ATRA (10⁻⁶ M) 48 h, eosinophil apoptosis was determined by flow cytometry; Annexin V was used to stain the early phase apoptotic cells and PI was used to stain the late phase cells. Concentration-dependent response (B) and time course (C) of eosinophil survival (n = 4–5). Eosinophil viability was assessed by the percentage of annexin V (−) and PI (−) cells. * p < 0.05 vs vehicle control.
then stained with Diff-Quick staining. Evidence of apoptotic morphology such as decreased cell size, nuclear condensation, and anucleation was assessed by light microscopy in a blind fashion. Nonapoptotic eosinophils were also assessed for the number of nuclear segmentations. Two hundred eosinophils were counted, and the percentage of eosinophils in each morphological change was calculated.

**Microarray hybridization**

After incubation with vehicle, 10^{-6} M 9-cis-RA or ATRA in 1% human serum albumin-coated plates, each 4 x 10^3 cells, for 4 h, eosinophils were lysed, and total RNA was isolated using Isogen (Nippon Gene) as per the manufacturer’s instructions. RNA was repurified with phenol-chloroform lysis and ethanol precipitation. For each cell culture condition, the RNA mixture was purified with phenol-chloroform lysis, and total RNA was isolated using Isogen (Nippon Gene) as per the manufacturer’s instructions. Concentrations of IL-5, IL-8, GM-CSF, vascular endothelial growth factor (VEGF), M-CSF, and MCP-1 were measured using an ELISA kit (R&D Systems), and colorimetric measurements were performed according to the manufacturer’s instructions. Concentrations of IL-5, IL-8, GM-CSF, vascular endothelial growth factor (VEGF), M-CSF, and MCP-1 were measured using an ELISA kit (R&D Systems), and colorimetric measurements were performed according to the manufacturer’s instructions.

**RT-PCR confirmation of gene microarray data**

For confirmation of expression patterns indicated by microarray analysis, 4 μg of total RNA was subjected to first-strand cDNA synthesis as described in Microarray hybridization. RT minus controls were included for each RT reaction. Primers and TaqMan probes for target genes were purchased from Applied Biosystems TaqMan Gene Expression Assays. Quantification of target cDNA and an internal reference gene (GAPDH) was performed in a final volume of 25 μl containing cDNA equivalent to 10–100 ng of total RNA, 12.5 μl of 1× TaqMan Universal PCR Master Mix, and 1.25 μl of 20× TaqMan Expression Assay reagent. Each sample was analyzed in triplicate. Thermal cycler conditions were 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C

Table I. Number of nuclear segmentations

<table>
<thead>
<tr>
<th></th>
<th>Live Cells (no. of nuclear segmentation)</th>
<th>Apoptotic Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Purified</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eosinophil</td>
<td>0.0 ± 0.0</td>
<td>90.7 ± 3.9</td>
</tr>
<tr>
<td>Vehicle</td>
<td>69.6 ± 8.0</td>
<td>27.3 ± 7.7</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.3 ± 0.1</td>
<td>80.9 ± 7.8</td>
</tr>
<tr>
<td>9-cis-RA</td>
<td>2.4 ± 1.9</td>
<td>76.2 ± 7.1</td>
</tr>
<tr>
<td>ATRA</td>
<td>3.8 ± 3.1</td>
<td>77.5 ± 6.1</td>
</tr>
</tbody>
</table>

* Data are expressed as the mean of three experiments ± SEM.
for 15 s and 60°C for 1 min. The comparative cycle threshold method of data analysis was used to analyze the data.

**Measurement of caspase 3 activity**

After treatment of eosinophils with 9-cis-RA and ATRA for 18 h, caspase 3 activity in these cells was assayed with an APOPCYTO colorimetric assay kit (MBL) according to the manufacturer's instructions.

**Statistical analysis**

For comparison of groups, ANOVA was used. If the ANOVA was significant, post hoc pairwise comparisons were conducted using Tukey's test, with the level of statistical significance taken as $p < 0.05$. The results are expressed as mean ± SEM.

**Results**

**Retinoid receptor profile of human peripheral blood eosinophils**

To provide a baseline for the interpretation of our studies, we first investigated the gene expression of all known RAR subtypes in human peripheral blood eosinophils using RT-PCR. The expression profile was compared with freshly isolated human monocytes. We found that human eosinophils expressed mRNA for all RAR subtypes. In contrast, RARβ was undetectable in human monocytes, consistent with a previous report (28). A similar pattern of RXR mRNA expression was observed between monocytes and eosinophils: RXRα and RXRβ, but not RXRγ, were expressed (Fig. 1A). On the basis of these results, the protein expression of RARs and RXRs on eosinophils was studied. Using Western blotting, we demonstrated that all three RAR isoforms, RXRα, and RXRβ were present in the lyses of eosinophils from two different donors (Fig. 1B). We also confirmed the protein expression by flow cytometry analysis on permeabilized cells. Similarly to the Western blotting, RARα, RARβ, RARγ, RXRα, and RXRβ were present in the lyses of eosinophils from two different donors (Fig. 1B). We also confirmed the protein expression by flow cytometry analysis on permeabilized cells. Similarly to the Western blotting, RARα, RARβ, RARγ, RXRα, and RXRβ were expressed in human eosinophil preparations (Fig. 1C). Because RXRγ mRNA was not detected by RT-PCR, RXRγ protein expression was not studied further.

**Table II. RA induced genes >2-fold over vehicle control samples and RA reduced genes <0.5-fold**

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>9-cis-RA/Vehicle</th>
<th>ATRA/Vehicle</th>
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<tbody>
<tr>
<td>M-CSF (M37435)</td>
<td>8.89</td>
<td>6.03</td>
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<tr>
<td>CD38 (D84276)</td>
<td>6.41</td>
<td>4.11</td>
</tr>
<tr>
<td>Vesicle-associated membrane protein 5 (BC017891)</td>
<td>5.65</td>
<td>4.06</td>
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<tr>
<td>ICAM-1 (CD54) (J03132)</td>
<td>3.54</td>
<td>2.95</td>
</tr>
<tr>
<td>Peroxiredoxin 6 (NM_004905)</td>
<td>3.32</td>
<td>2.90</td>
</tr>
<tr>
<td>Cathepsin D (M11233)</td>
<td>3.27</td>
<td>2.80</td>
</tr>
<tr>
<td>Selectin L (NM_000655)</td>
<td>3.21</td>
<td>2.64</td>
</tr>
<tr>
<td>Putative lymphocyte Gα-G1 switch gene (M72885)</td>
<td>2.89</td>
<td>2.43</td>
</tr>
<tr>
<td>Peripheral myelin protein 22 (PMP-22) (D11428)</td>
<td>2.86</td>
<td>2.30</td>
</tr>
<tr>
<td>IFN-α/β receptor 2 (long form) (X89772)</td>
<td>2.67</td>
<td>2.10</td>
</tr>
<tr>
<td>VEGF (AF022375)</td>
<td>2.60</td>
<td>1.64</td>
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<tr>
<td>FK506-binding protein 2 (FKBP2) (M61238)</td>
<td>2.47</td>
<td>1.82</td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor-like 2 (CCRL2) (AF014958)</td>
<td>2.40</td>
<td>1.78</td>
</tr>
<tr>
<td>IFN regulatory factor 1 (IRF1) (X14454)</td>
<td>2.38</td>
<td>2.10</td>
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<tr>
<td>Galectin-9 (Z49107)</td>
<td>2.33</td>
<td>1.54</td>
</tr>
<tr>
<td>IFN (α, β, and γ) receptor 2 (IFNAR2) (L41944)</td>
<td>2.24</td>
<td>1.67</td>
</tr>
<tr>
<td>Adipophilin (X97324)</td>
<td>2.15</td>
<td>0.97</td>
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<tr>
<td>Glutathione S-transferase A2 (U77604)</td>
<td>2.01</td>
<td>1.45</td>
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<tr>
<td>Synaptic vesicle membrane protein VAT-1 homolog (U18009)</td>
<td>2.01</td>
<td>1.18</td>
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</tbody>
</table>

For 15 s and 60°C for 1 min. The comparative cycle threshold method of data analysis was used to analyze the data.

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9-cis-RA and ATRA markedly delay eosinophil apoptosis

It has been reported that RAs (10^{-6} M) regulate survival in several types of cells. To investigate the functional role of RAs, we first examined the capacity to modulate eosinophil apoptosis that spontaneously occurs after culturing these cells. Eosinophil apoptosis was determined by flow cytometry; Annexin V was used to stain the early phase apoptotic cells, while PI was used to stain the late phase cells. Purified human blood eosinophils were incubated with the natural ligand for RARs and RXRs (9-cis-RA and ATRA, 10^{-6} M) or vehicle control (0.1% DMSO) for 48 h. The percentage
of cells undergoing apoptosis was dramatically decreased in eosinophils incubated with 9-cis-RA or ATRA compared with vehicle control (Fig. 2A). 9-cis-RA and ATRA delayed eosinophil apoptosis in a dose-dependent manner and occurred around their physiological concentrations (10^{-6} M to 10^{-8} M) (Ref. 29 and Fig. 2B). Of note, their ability to delay apoptosis almost equaled that of IL-5 (1 ng/ml), a critical cytokine mediating eosinophil survival. As shown in Fig. 2C, eosinophils incubated with RAs displayed almost 80% viability at 72 h after incubation (vehicle, 12.0 ± 4.1%; 9-cis-RA, 81.9 ± 3.5%; and ATRA, 78.0 ± 3.8%).

Morphological appearance of eosinophils incubated with RAs

To assess the morphological evidence of apoptosis and cell activation, eosinophils were cultured for 48 h with vehicle alone, IL-5 (1 ng/ml), 9-cis-RA (10^{-6} M), or ATRA (10^{-6} M) and were observed by light microscopy (Fig. 3). Reflecting spontaneous apoptosis, eosinophils with apoptotic morphology (cytoplasmic and nuclear chromatin condensation, and anucleation) were seen after they were cultured with vehicle alone. In contrast, eosinophils cultured with IL-5, 9-cis-RA, or ATRA avoided apoptotic changes and, interestingly, several nonapoptotic cells had more than three nuclear lobes. Because nuclear hypersegmentation is induced by eosinophilopoietic cytokines and indicates the increased life span (30), the number of nuclear segmentations besides apoptotic change was quantified and is shown in Table I. In line with the flow cytometric studies, after culture for 48 h, apoptotic eosinophils were decreased by IL-5 or RAs compared with vehicle alone. Of note, incubation with IL-5 or RAs induced a significant increase in the number of eosinophils with hypersegmented nuclei, while most of the freshly purified peripheral blood eosinophils or vehicle-cultured live eosinophils showed bisegmented nuclei. These data provide multiple lines of evidence demonstrating prolonged survival induced by RAs.

Effect of pharmacological agonists and antagonists on eosinophil survival

To determine which of the two types of RARs is involved in the antiapoptotic effect, we used the specific agonists for each receptor: RAR-selective agonist TTNPB (31) and RXR-selective agonist HX630 (32). Eosinophils were cultured in the presence of either TTNPB or HX630 for 48 h, and cell viability was assessed by flow cytometry staining with annexin V and PI. Interestingly, both TTNPB and HX630 delayed the eosinophil apoptosis, although HX630 had a less potent effect (Fig. 4A). The cell viability of vehicle, TTNPB (10^{-6} M), and HX630 (10^{-6} M) was 15.2 ± 5.5%, 73.4 ± 3.4%, and 28.8 ± 5.7%, respectively. These results indicate that both RARs and RXRs (mainly RARs) are involved in the effect of RAs to delay eosinophil apoptosis. Next, we examined the effect of receptor blockade on RA-induced eosinophil survival. As shown in Fig. 4B, pretreatment with RARα-specific antagonist Ro41-5253 nearly completely inhibited the effect of ATRA at the

![FIGURE 5](http://www.jimmunol.org/)

The effect of neutralizing anti-IL-5 or anti-GM-CSF Abs on RA-induced eosinophil survival. Eosinophils were stimulated with 10^{-6} M RA along with neutralizing anti-IL-5 or anti-GM-CSF (20 μg/ml) Abs for 48 h, and eosinophil viability was then determined by flow cytometry with the percentage of annexin V (−) and PI (−) cells (n = 4). Eosinophil survival was significantly prolonged by culturing with IL-5 (1 ng/ml) or GM-CSF (0.1 ng/ml) and these effects were completely blocked by each neutralizing Ab. In contrast, neutralizing anti-IL-5 or anti-GM-CSF (20 μg/ml) Abs failed to reverse the effect of RAs. *, p < 0.05.

![FIGURE 6](http://www.jimmunol.org/)

Eosinophils were cultured with 9-cis-RA (10^{-6} M) or ATRA (10^{-6} M) for 48 h. The cell culture supernatants were then subjected to a membrane array containing 23 different cytokine/chemokine Abs. 9-cis-RA and ATRA induced eosinophils to release MCP-1 (arrows). Results are representative of n = 2.
concentration of $10^{-5}$ M (70.1% inhibition). In contrast, the anti-apoptotic effect of 9-cis-RA was partially blocked by Ro41-5253 at $10^{-5}$ M (26.9% inhibition). Therefore, it is suggested that RARα activation is important for RA-induced eosinophil survival, although RXR activation can partially compensate for the effect.

**Identification of RA-dependent transcriptional targets by gene microarray**

In an attempt to identify genes that are associated with RA-induced eosinophil survival, we next determined the expression levels of eosinophil transcripts using a microarray following stimulation with vehicle, 9-cis-RA ($10^{-6}$ M), or ATRA ($10^{-6}$ M) for 4 h. Additional longer time points were prohibited by the difficulty of obtaining subjects to harvest the required amount of RNA for the microarray. Approximately 444 genes were detected on the chip containing 747 genes (raw data are available at the Gene Expression Omnibus site as cited in Materials and Methods). There was little variation between the samples treated by 9-cis-RA and ATRA. The main results are indicated in Table II. The comparison between vehicle control and 9-cis-RA, or vehicle control and ATRA, identified an increase (>2-fold change over control) in 19 and 11 transcripts, respectively. The number of decreased transcripts (<0.5-fold) resulting from 9-cis-RA or ATRA exposure was 19 and 8 transcripts, respectively. In these transcripts, the expression changes relative to vehicle control were smaller in with ATRA compared with 9-cis-RA, suggesting that 9-cis-RA induced gene transcription more effectively than ATRA. Of note, among the 10 genes with the biggest decrease relative to vehicle control, we found an apoptosis-related gene, caspase 3 (9-cis-RA, 0.33-fold decrease and ATRA, 0.39-fold decrease).

**RAs induced production of M-CSF, VEGF, and MCP-1, but they were not involved in antiapoptotic effect**

RAs affect cytokine, chemokine, and growth factor production from several types of cells. Because of the strong efficacy of RAs and the potential of eosinophil to produce IL-5 and GM-CSF, critical promoters of eosinophil survival (2), we examined whether RAs prolong survival by autocrine production of IL-5 or GM-CSF using neutralizing Abs. However, cotreatment of neutralizing anti-IL-5 or anti-GM-CSF Abs failed to reverse the effect of RAs (Fig. 5). We also found that IL-3, an eosinophil survival-promoting cytokine (2), was not involved in RA-induced cell survival using a neutralizing anti-IL-3 Ab ($n = 3$; data not shown). Moreover, neither IL-5 nor GM-CSF in the supernatants of eosinophils cultured with RAs for 24 h was detected by ELISA (vehicle control, 65.1 ± 7.1%; VEGF, 62.2 ± 7.1%; and ATRA, 63.6 ± 7.4%; vehicle control, 65.5 ± 8.5%; and combination of M-CSF, VEGF, and MCP-1, 63.2 ± 8.4%; $n = 3$). These results indicated that RAs triggered eosinophils to produce M-CSF, VEGF, and MCP-1, but they were not involved in inhibition of spontaneous eosinophil apoptosis.
RA derivatives on human eosinophils. Our study adds additional information on the potential role of vitamin A in regulating immune responses.

Nuclear hormone receptors mediate the important biological function on eosinophils. We previously reported that human eosinophils expressed peroxisome proliferator-activated receptor γ (PPARγ), a heterodimer partner of RXR, and that stimulation of eosinophils with a synthetic PPARγ agonist inhibited IL-5-induced eosinophil survival by leading apoptosis (22). Based on previous reports that the PPARγ agonist-induced apoptotic effect was increased by cotreatment with the agonist for RXR (36) or with 9-cis-RA (37), we initially speculated that PPARγ/RXR activation by each agonist synergistically induced eosinophil apoptosis. However, contrary to our expectation, a preliminary study revealed that cotreatment of eosinophils with 9-cis-RA and a synthetic PPARγ agonist prolonged cell survival. In this study, because of the strong efficacy of RAs to inhibit eosinophil apoptosis comparable to that of eosinophilopoietic cytokines such as IL-3, IL-5, and GM-CSF, we investigated the ability of RAs on eosinophils to trigger antiapoptotic protein secretion in cell culture supernatants, although they were not involved in the effect of RAs. Furthermore, the intracellular expression of Bcl-2 and Bcl-xL, which are involved in the process of cytokine-induced inhibition of eosinophil apoptosis (38), were not altered by stimulation with 9-cis-RA or ATRA (our unpublished data). Taken together with the pharmacologic manipulations with the specific agonists and antagonists, the evidence could support the hypothesis that RAs directly inhibit eosinophil spontaneous apoptosis through activation of both RARs and RXRs.

Because little is known about the mechanism behind the antiapoptotic effect of activated nuclear hormone receptors on eosinophils, we used a gene microarray to search for RA-responsive antiapoptotic targets. Among the several transcripts responsive to both 9-cis-RA and ATRA, down-regulation of caspase 3 was screened as the most likely candidate responsible for the eosinophil survival-promoting activity. Spontaneous eosinophil apoptosis involves Bax translocation to the mitochondria, cytochrome c release, and perturbation of the mitochondrial membrane followed by activation of caspases (39). In the case of IL-5-induced eosinophil survival, it is mediated by inhibition of Bax translocation and the consequent activation of the caspase pathway. We were able to show that the inhibition of spontaneous caspase 3 activation was nearly the same extent as that of eosinophilopoietic cytokine IL-5. Taken together, our data suggest that the caspase 3-mediated spontaneous apoptotic process is, at least in part, involved in RA-induced prolonged eosinophil survival. In contrast, we cannot rule out the possibility that the effects of RAs are mediated by an additional molecular target(s), as indicated in the results from the gene array. For example, peroxiredoxin 6, ranked as the fifth most-increased transcript both in 9-cis-RA- and ATRA-stimulated eosinophils, uses glutathione to catalyze the reduction of reactive oxygen species and protects from reactive oxygen species-induced cytotoxicity in different cell types (40). The second most-decreased transcript, fox, is known to be an AP-1 transcription factor and is involved in the regulation of myeloid differentiation (41), but it is not clear whether it also has other effects on human eosinophils. Thus, further studies are necessary to clarify the involvement of these molecules and/or alternative mechanisms, but such studies will definitely provide interesting insights into the physiological importance of RAs and these molecules on eosinophil survival.

RAs are known to be important regulators of granulopoiesis. Dietary vitamin A-deficient mice exhibited abnormal expansion of myeloid cells probably due to impaired spontaneous apoptosis (42), and mice genetically deficient in both RARα and RARγ displayed an in vitro block in granulocyte differentiation (43). Thus, it is now widely accepted that ATRA is a clinically useful compound that induces terminal granulocyte differentiation and apoptosis of most acute promyelocytic leukemia cells. In addition, retinoids can alter lineage commitment: ATRA suppresses erythroid maturation of normal pluripotent progenitor cells, forcing differentiation toward the neutrophil lineage (44). It was reported that ATRA inhibited eosinophil differentiation of CD34+ cord blood cells (45) and bone marrow mononuclear cells obtained from the hypereosinophilic syndrome (46). In line with these observations, previous studies with eosinophil-committed cell lines such as HL-60 (47) and AML14.3D10 (48) have also indicated the inhibition of eosinophil differentiation by RAs. In contrast to these data that point out the inhibition of survival and differentiation in immature eosinophils by RAs, here we clearly demonstrated that RAs prolong survival in terminal differentiated eosinophils in vitro. This effect was quite reproducible, as it was consistently observed using eosinophils from many different donors. One explanation for this difference is that the effect of RAs is cell type specific and could be changed according to the stage of differentiation. The plasma concentration of RAs has been reported to reach 10⁻⁸ M (29), which is sufficient to stimulate eosinophil survival, as shown in our study; hence, RAs may play a role in the regulation of eosinophil homeostasis.

This work also led us to consider the potential role of RAs in allergic inflammatory diseases, namely, bronchial asthma. The majority of the body’s vitamin A is stored in the liver; however, many other organs including the lung have a large concentration of vitamin A (6). Vitamin A derivatives are known to influence the development, maintenance, differentiation, and regeneration of
lungs epithelial cells (49); hence, it may play a central role in the development of allergic airways. Interestingly, Shoeyev et al. (50) showed that asthmatic rats increased vitamin A utilization by repleted allergen inhalation. Furthermore, it was reported that the serum vitamin A concentration in asthmatic patients in both developing and developed countries was significantly lower than that in normal controls (51, 52). Thus, these studies suggest increased vitamin A utilization in allergic airway inflammation.

Eosinophils contribute to persistent airway inflammation, which leads to airway structural changes called airway remodeling, including subepithelial fibrosis, hyperplasia of mucus glands, myofibroblast and smooth muscle proliferation, and vascular changes (2). In the course of the screening procedure with a gene microarray and a cytokine array, we discovered that RAs induced VEGF and MCP-1 production in cell culture supernatants. In the asthmatic airway, they are produced in abundance and are thought to be involved in the allergic inflammatory processes by activation of their target cells, especially vascular endothelial cells and monocytes/macrophages (53, 54). Another intriguing observation was that RAs induced hypersegregation of eosinophil nuclei, a morphological abnormality often observed in cells from bronchoalveolar lavage fluid in eosinophil pneumonia (30, 55). This evidence raises the possibility of RAs as a significant contributor to sustaining the heightened eosinophilic inflammatory response and of anti-RARs as a novel therapeutic strategy in asthma and other allergic inflammatory diseases. Efforts are currently under way to clarify the pathophysiological significance of RAs using an in vivo model.

In conclusion, our current data provide novel insights into the roles of vitamin A in eosinophil homeostasis. Physiological levels of RAs have a potent inhibitory effect on eosinophil spontaneous apoptosis and might facilitate sustainment of allergic inflammation. Our finding is potentially of therapeutic importance, as an anti-RAR/RXR strategy would not only be the antiapoptotic effect on eosinophils but also the action of removing the potential role of vitamin A utilization in allergic airway inflammation.

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