Increased Expression and Activation of CD30 Induce Apoptosis in Human Blood Eosinophils

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Increased Expression and Activation of CD30 Induce Apoptosis in Human Blood Eosinophils

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Eosinophils are one of the major effector cells in asthma, and controlling the number and survival of eosinophils might attenuate the severity of asthma. This result could be achieved by inducing eosinophil apoptosis. Apoptosis allows the removal of cells without inducing an inflammatory response. Our knowledge of the factors involved in regulating eosinophil apoptosis remains limited. CD30 molecule has been associated with T cell-negative selection and in TCR-mediated apoptosis. In this study we examined the expression and role of CD30 in apoptosis of human blood eosinophils. Percentage of apoptotic eosinophils was determined by annexin V-propidium iodide labeling, and CD30 expression was examined by flow cytometry. Spontaneous apoptosis was induced by serum deprivation, and survival was conferred by incubating cells with 10% FBS and IL-5. CD30 surface expression was up-regulated in eosinophils incubated for 24 h as compared with freshly isolated eosinophils, and both CD30 expression and eosinophil apoptosis increased in a time-dependent manner. We also measured CD30 mRNA expression by quantitative real-time RT-PCR and determined that CD30 transcripts increased in eosinophils undergoing apoptosis only under serum deprivation conditions. The agonistic CD30 Abs, Ber-H8 and HeFi-1, significantly enhanced eosinophil apoptosis. FBS and IL-5 failed to inhibit or suppress the CD30 agonistic-induced apoptosis. These data support the role of CD30 activation in eosinophil apoptosis. This research will help in furthering our understanding of eosinophil apoptosis and therefore might contribute to the development of better therapeutic modalities in the treatment and/or cure of allergic inflammation in bronchial asthma. The Journal of Immunology, 2004, 173: 2174–2183.

Asthma is a complex chronic inflammatory disease of the lungs that results in recurrent wheezing episodes, chest tightness, coughing, and shortness of breath (1). The asthmatic attack consists of early and late allergic responses. Histamine, leukotriene C_{4}, and PGD_{2} released by mast cells characterize the early response that occurs within minutes after allergen exposure, thereby bringing about bronchoconstriction, vasodilation, and mucus buildup (2, 3). Late phase response occurs 3–9 h after early response and may last for days if not treated. Late phase asthma is marked by elevated levels of IL-4, IL-5, IL-13, IL-16, eosinophil chemotactic factor, TNF-α, and platelet-activating factor that increases endothelial cell adhesion and recruits inflammatory cells, mainly eosinophils and neutrophils, to the bronchial tissue (4, 5).

Eosinophils are one of the most important inflammatory cells in asthma. Many studies have demonstrated a strong association between airway eosinophilic inflammations and the development of lung pathology observed in asthma (6). Eosinophilic inflammation in asthma is considered the major contributor to the structural changes in the airways of individuals with chronic asthma, including fibrosis, thickening of the airway smooth muscle layer due to hypertrophy, and hyperplasia of goblet cells (7, 8). A major player in contributing to eosinophilia in asthma is the hypersecretion of IL-5 (9). It has been long established that IL-5 is vital in promoting eosinophil proliferation, differentiation, activation, chemotaxis, and survival (10, 11). Eosinophils in inflammation tissue of patients with allergic asthma display a prolonged half-life of a few weeks as compared with the normal 1- to 2-day half-life. IL-5, along with other cytokines such as IL-3 and GM-CSF, are up-regulated in the asthmatic airways and are primarily responsible for mediating this lengthy life span of eosinophils in asthmatic individuals (12, 13).

Apoptosis, or programmed cell death, is an essential process for maintaining homeostasis, especially in the immune system. Apoptosis is characterized by nuclear fragmentation, chromatin condensation, translocation of phosphatidylinerine, and the formation of apoptotic bodies that are eventually phagocytosed by macrophages, thus preventing any local inflammation as is the case in necrosis (14, 15). Eosinophil apoptosis can be induced in response to activation of death-inducing Fas receptors (CD95), or in response to cellular stress factors, including serum and/or growth factors withdrawal, and DNA damage (16, 17). Conversely, eosinophil survival is mainly mediated by three hematopoietins, IL-3, IL-5, and GM-CSF (12, 16). IL-5 up-regulates antiapoptotic proteins and induces the activation prosurvival signaling pathways within eosinophils (18–20).

In our preliminary studies using DNA microarrays analysis we observed a significantly increased expression of CD30 molecules in human blood eosinophils under certain conditions (data not shown). This was an interesting finding because previous studies failed to identify detectable levels of CD30 expression in eosinophils (21, 22), and identification was only very recently reported after completion of our report (23). CD30, originally identified as Ki-1 Ag on Reed-Sternberg cells of Hodgkin’s lymphoma (24), is a 120-kDa heavily glycosylated protein member of the TNFR superfamily that includes TNFR-I, TNFR-II, Fas, CD40, and other receptors (25, 26). CD30 molecule consists of an 18-amino acid

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(aa) leader peptide, followed by a 365-aa-extractable polypeptide domain, a 24-aa transmembrane segment, and a 188-aa cytoplasmic domain. The full-length CD30 molecule is a 120-kDa heavily glycosylated protein. The extracellular portion of CD30 can be proteolytically cleaved by zinc-metalloprotease to produce an 85/90 kDa soluble form (27–29). CD30 activation participates in a variety of biological effects depending on cell type, ranging from cell proliferation and activation to apoptosis (20–32).

We report that eosinophils express CD30 molecules and that this expression is up-regulated in eosinophils undergoing apoptosis. Further we demonstrate that CD30 activation induces eosinophils to undergo apoptosis and that IL-5 and FBS failed to protect eosinophils from CD30-induced apoptosis.

Materials and Methods

Human subjects

Male and female subjects, 19- to 65-years-old, were recruited from our existing database at Creighton University Center for Allergy, Asthma and Immunology (Omaha, NE). All subjects were either normal or were patients with mild-to-moderate allergic asthma. Normal subjects were individuals who had no diagnosed allergies based on negative skin tests, absence of allergy symptoms, and no history of wheezing and asthma. Mild allergic asthma patients were defined based on wheezing and asthma symptoms, which were reversible with bronchodilator. The values for forced expiratory volume in 1 s for patients with allergic asthma ranged from 80% to 100%. Subjects were not taking any medication until a final volume of 50 µl. Reaction buffer containing 10 mM DTT was added to each sample. Substrate (5 µl of DEVD substrate for caspase-3 enzyme, IEHT substrate for caspase-8 enzyme, LEHD substrate for caspase-9 enzyme) was added to wells, followed by 2 h incubation at 5% CO2 and 37°C. Absorbance development was read at 405 nm in a microtiter plate reader and OD results reported after the subtraction of the background.

RNA extraction and RT-PCR

Eosinophils were harvested by centrifugation at 300 × g for 5 min and total RNA was isolated using the RNAqueous-4PCR kit from Ambion (Austin, TX) following the procedure recommended by the supplier. RT-PCR was performed using the OneStep RT-PCR kit from Qiagen (Valencia, CA) and ribosomal protein RPL13A as an internal control. In summary, isolated RNA was reverse transcribed using Omniscript and Sensiscript Reverse Transcriptase (Qiagen) for 40 min at 50°C. Reverse transcription was inactivated by heating samples up to 95°C for 15 min, which simultaneously activates the HotStarTaq DNA polymerase. PCR was carried for 20 cycles (RPL13A) and 30 cycles (CD30) of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The optimal number of PCR cycles for CD30 expression (30 cycles) and for the internal ribosomal control RPL13A (20 cycles) were determined by initially performing PCR cycles and establishing the linearity curve for each gene (data not shown). DNA contamination was also tested by including a PCR control reaction in every experiment. Primer sequences were synthesized as follows: CD30 (sense) 5'-GTTGAGGCGAGCAACAGATGG-3', CD30 (antisense) 5'-GAGATGAGTGACTTGATCCTGG-3', RPL13A (sense) 5'-GGCCATCGTGGCTAAACAGG-3', RPL13A (antisense) 5'-CGTCTTCCGGCGCTTTC-3'. The amplification products were separated on a 1% agarose gel at 75 V for 2 h. The bands were then visualized under UV light after staining with ethidium bromide. After exposure to UV light, the density of DNA bands was determined using Kodak EDESS 290 software system and the density values for CD30-specific amplification products were normalized against those for an internal control.

Quantitative real-time RT-PCR

For the real-time quantitative RT-PCR (qRT-PCR)3 experiments, RNA was isolated using TRI REAGENT from MRC (Cincinnati, OH) following the single-step method provided by the supplier. qRT-PCR was performed using the Quant iTech SYBR Green RT-PCR kit from Qiagen and GAPDH as an internal control. Experiments were performed by using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). As previously described, isolated RNA was reverse transcribed using Omniscript and Sensiscript Reverse Transcriptases (Qiagen), for 40 min at 50°C. Reverse transcription was inactivated by heating samples up to 95°C for 15 min, which simultaneously activates the HotStarTaq DNA polymerase. PCR was carried for 40 cycles of 95°C for 2 s, 55°C for 20 s, and 72°C for 30 s. DNA contamination was also tested by including a PCR control reaction in every experiment. Primer sequences were synthesized as follows: CD30 (sense) 5'-CTGCGACCATAGGAAACAAGACCGT-3', CD30 (antisense) 5'-CCGGAACTCCCAACAGCTACCTTTA-3'; GAPDH (sense) 5'-GAATTCTGGCTACAGGACAGGTTG-3', GAPDH (antisense) 5'-TCTTCTCCCTGGTCTGCTGGTG-3'. The amplification products were analyzed using the ABI Sequence Detection System 1.1 software (Applied Biosystems) to determine relative quantitative (RQ) levels of CD30 mRNA expression in comparison to those of freshly isolated eosinophils (0 h).

Detection of CD30 surface expression

Eosinophils were resuspended in PBS supplemented with incubation buffer (1% BSA, 3% FBS, and 0.05% sodium azide). Cells were then incubated with either the incubation buffer only, FITC-conjugated anti-human CD30

3 Abbreviations used in this paper: qRT, real-time quantitative RT-PCR; RQ, relative quantification; TRAF, TNFR-associated factor.
Abs, or FITC-conjugated isotype-matching IgG (2 μg/100 μl) obtained from Santa Cruz Biotechnology (Santa Cruz, CA) for 45 min on ice. After washing twice with the incubation buffer, cells were fixed with 4% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ).

**CD30 activation assay**

Abs used were the CD30 agonistic Abs HeFi-1 (a kind gift of the National Cancer Institute, Bethesda, MD) and Ber-H8 (BD Pharmingen), CD30 antagonistic Abs Ber-H2 and Ki-1, or IgG isotype control Ab (Santa Cruz Biotechnology).}

**FIGURE 1.** Effect of IL-5 and FBS on eosinophil apoptosis. Neither IL-5 nor FBS alone provide eosinophils with sufficient protection against apoptosis. Purified blood eosinophils were either processed fresh or incubated for 24 h in RPMI 1640 medium supplemented with different concentrations and combinations of IL-5 and heat-inactivated FBS, then analyzed for apoptosis using annexin V-propidium iodide. Annexin V-positive and annexin V-propidium iodide double-positive cells were considered apoptotic. Data represent mean ± SEM of n = 3; *, p < 0.05 and **, p < 0.01 as compared with baseline apoptosis of each experimental group (no FBS, 1% FBS, and 10% FBS).

**FIGURE 2.** Apoptosis of eosinophils isolated from different subject groups. Eosinophils were purified from blood collected from normal (nonallergic/nonasthmatic), allergic (atopic/nonasthmatic), or allergic asthmatic (atopic asthmatic) individuals. Purified eosinophils were incubated for 48 h in RPMI 1640 medium supplemented with either 1% FBS or 10% FBS and 15 ng/ml IL-5, then analyzed for apoptosis using annexin V-propidium iodide. Annexin V-positive and annexin V-propidium iodide double-positive cells were considered apoptotic. Data represent mean ± SEM of n = 5; ***, p < 0.001.
Abs were suspended in PBS at a concentration of 20 μg/ml, and 500 μl/well were transferred to flat-bottom 24-well plates (Costar, Cambridge, MA). After Ab immobilization at 4 °C for 48 h, wells were washed three times with PBS containing 1% BSA and blocked with 1 ml of 1% BSA at 4 °C overnight. Eosinophils (1 ml of 1 × 10^6 cells/ml) were added to each well. After incubation at 37 °C for 12 or 24 h, eosinophils were collected and apoptosis was measured by flow cytometry.

Statistical analysis

Values for all measurements are expressed as the mean ± SEM. One-way ANOVA was used to determine the difference among various experimental groups. Statistical difference between two groups was performed by the Student t test. Values of p < 0.05 were considered as statistically significant.

Results

Spontaneous apoptosis, the in vitro system, and IL-5 and FBS protection

We established a model of human blood eosinophil survival and apoptosis using serum withdrawal as a method to induce eosinophil spontaneous apoptosis. Baseline percentage of apoptotic eosinophils in purified samples was low at 3.33 ± 0.47% as measured by the annexin V-propidium iodide technique. Initial studies were conducted using different concentrations and combinations of FBS and IL-5 (Fig. 1). Our experiments revealed that eosinophils, when incubated in RPMI 1640 medium supplemented with 15 ng/ml IL-5 and 10% heat-inactivated FBS, remain protected from apoptosis at least up to 72 h. In comparison, a significant percentage of eosinophils incubated with RPMI 1640 supplemented only with 1% FBS underwent apoptosis (72-h incubation, data not shown). Despite the fact that both IL-5 and FBS individually provided some significant levels of protection for eosinophils against spontaneous apoptosis, neither, however, provided complete protection when added individually in our initial experiments (Fig. 1). In the absence of IL-5, there was no significant effect of 1% FBS on the survival of eosinophils. However, 10% FBS decreased apoptosis of eosinophils from 60.14 ± 2.22% to 42.12 ± 4.24% (Fig. 1). In the absence of FBS, IL-5 significantly decreased eosinophil apoptosis at both 1 and 15 ng/ml concentrations (45.08 ± 1.66% and 27.55 ± 3.97%, respectively). Addition of either 1 or 10% FBS with IL-5 further decreased the apoptosis. In the presence of either 1 or 10% FBS, effect of 1 ng/ml IL-5 was similar to that of 15 ng/ml IL-5 (Fig. 1).

Based on these observations and limitation on the number of freshly isolated eosinophils, we restricted our model of eosinophil apoptosis to three experimental groups; freshly isolated eosinophils, eosinophils incubated with 1% FBS, and eosinophils incubated with 10% FBS and 15 ng/ml IL-5. Additional experiments performed by incubating the eosinophils for 24 h demonstrated that 53.1 ± 3.3% of eosinophils incubated with only 1% FBS have undergone apoptosis. Adding 15 ng/ml IL-5 and 10% FBS to RPMI 1640 medium almost completely suppressed spontaneous eosinophil apoptosis observed under serum withdrawal, as only...
11.5 ± 1.1% eosinophils were apoptotic after 24 h. We also observed no significant difference in apoptosis levels between eosinophils isolated from normal, allergic, or allergic asthmatic individuals (Fig. 2).

Elevated caspase activity in eosinophils undergoing apoptosis

Caspases are a family of cysteine proteases that are regarded as one of the main intracellular effector systems of apoptosis. Caspase-8 and caspase-9 are considered to be the “initiator” caspases, whereas caspase-3 is the “executor” caspase (35). We measured caspase activity in fresh eosinophils, and in eosinophils incubated for 24 h with either 1 or 10% FBS and 15 ng/ml IL-5. Caspase-3, caspase-8, and caspase-9 activities were minimal in freshly isolated eosinophils as well as in the eosinophils incubated with 10% FBS and 15 ng/ml IL-5 for 24 h. In eosinophils incubated for 24 h with only 1% FBS, there was a 3-fold increase in both caspase-8 and caspase-9 activities, and a 12-fold increase in the activity of executioner caspase-3 (Fig. 3).

CD30 mRNA and surface expression in eosinophils

We first examined the CD30 mRNA expression in human blood eosinophils by RT-PCR. Eosinophils expressed CD30 mRNA under all experimental conditions (Fig. 4a). CD30 mRNA expression appeared to increase in eosinophils incubated for 24 h. We performed qRT-PCR to accurately determine CD30 mRNA expression levels, and results are reported in RQ units as compared with freshly isolated cells at 0 h (RQ = 1.0) (Fig. 4, b and c). After 12-h incubation, CD30 mRNA expression significantly increased in eosinophils incubated with only 1% FBS (RQ = 2.3 ± 0.24), whereas expression virtually remained the same in eosinophils incubated with 10% FBS and 15 ng/ml IL-5 (RQ = 1.06 ± 0.16) (Fig. 4b). After 24-h incubation, CD30 mRNA expression maintained its high levels in eosinophils incubated with 1% FBS (RQ = 2.6 ± 6.4), however, it was slightly lower in eosinophils incubated with 10% FBS and 15 ng/ml IL-5 (RQ = 0.67 ± 0.1) (Fig. 4c).

The difference in mRNA expression between fresh cells and eosinophils incubated with 10% FBS and IL-5 for 24 h is <1-fold (RQ = 1.0 vs RQ = 0.67), and was not significantly different.

We next examined the expression of surface CD30 molecules on human blood eosinophils by flow cytometric analysis. Low percentage of freshly isolated eosinophils expressed CD30 (3.6 ± 3.1%) as compared with the eosinophils that were incubated with 10% FBS and 15 ng/ml IL-5 for 24 h (14.6 ± 5.4%) (Fig. 5). Interestingly, a high percentage of eosinophils (27.2 ± 5.2%) were CD30-positive when incubated with only 1% FBS for 24 h (Fig. 5).

Caspase-8 and caspase-9 are considered to be the one of the main intracellular effector systems of apoptosis. Caspases are a family of cysteine proteases that are regarded as executioner caspase (35). We measured caspase activity in fresh eosinophils, and in eosinophils incubated with 1% FBS and 15 ng/ml IL-5 for 24 h. In eosinophils incubated for 24 h with only 1% FBS, there was a 3-fold increase in both caspase-8 and caspase-9 activities, and a 12-fold increase in the activity of executioner caspase-3 (Fig. 3).

Induction of eosinophil apoptosis by CD30 Abs

To investigate the effect of CD30 activation on eosinophil apoptosis, we used the CD30 agonistic Abs, Ber-H8 and HeFi-1 (36). Ber-H8 and HeFi-1, at a concentration of 20 μg/ml, induced a significant elevation in eosinophil apoptosis as compared with the effect observed with the control groups. Initial experiments also revealed that Ber-H8 and HeFi-1 still induced eosinophil apoptosis at lower concentrations of 5 and 10 μg/ml, but were less effective as compared with 20 μg/ml (data not shown). Ber-H8 Abs, however, were the most effective in inducing eosinophil apoptosis at 12 and 24 h postincubation (Figs. 7 and 8). Under serum-deficient conditions, Ber-H8 induced the apoptosis of 62 ± 3.25% of eosinophils at 12 h as compared with 29.7 ± 4.9% in the control group without Ab, an increase of ~100% in apoptosis (Fig. 7a).

The percentage of apoptotic eosinophils incubated with Ber-H8 increased to 81.6 ± 5.5% after 24 h, whereas only 56.7 ± 4.1% were apoptotic in the control group (Fig. 7b). In comparison, CD30 activation with HeFi-1 Abs resulted in the apoptosis of 50.8 ± 3.7% and 69.1 ± 4.4% of eosinophils at 12 h and 24 h, respectively (Fig. 7, a and b).

Additional experiments revealed that IL-5 and FBS failed to protect eosinophils from the CD30-induced apoptosis (Fig. 8). In fact, Ber-H8 Abs induced 33.3 ± 3.2% and 49.3 ± 3.7% of eosinophils to undergo apoptosis at 12 h and 24 h, respectively, a
very significant and noticeable increase from the 8 ± 1.9% and 11.7 ± 2.4% observed in the control group (Fig. 8). HeFi-1 Abs were slightly less effective as they induced only 23.4 ± 2.9% and 35.1 ± 2.7% of eosinophils to undergo apoptosis at 12 h and 24 h, respectively. Because we used same dose of both Abs and we observed that Ber-H8 always elicited greater response than that by the HeFi-1 Ab, this might suggest an increased binding and/or affinity of Ber-H8 to CD30. The statistically significant difference between the two groups was only observed after 24-h incubation in the presence of IL-5 and FBS (Fig. 8b). No significant change in eosinophil apoptosis was observed in the Ber-H2, Ki-1, and the IgG control groups.

Discussion
Bronchial asthma is a chronic disease of the airways that affects the quality of life of millions and is becoming a major health concern in most of the industrialized countries. Airway hyperresponsiveness, blood, and tissue eosinophilia, and Th2 cells are the hallmarks of bronchial asthma. Eosinophilic inflammation in asthma is regarded as a major contributor to the structural changes observed in the airways of chronic asthma patients, including fibrosis and thickening of the airway smooth muscle layer (1–3). Strong evidence exists and supports the observation that the severity of airway hyperresponsiveness correlates with the degree of eosinophilia and sloughing of the airway epithelial cells. Eosinophilia is attributed to three main factors: increased eosinophil production, recruitment of eosinophil to airway tissue, and prolonged eosinophil survival. Eosinophils remain one of the prime targets in the treatment of airway diseases. Therefore we believe that inducing eosinophil apoptosis might provide a powerful tool to target and reduce the number of eosinophils present in the airways of asthmatics.
In this study we demonstrated that 10% FBS and 15 ng/ml IL-5 provided sufficient protection for eosinophils against spontaneous apoptosis observed under serum deprivation conditions. Data obtained from this study have revealed that whereas IL-5 and FBS combined suppressed eosinophil apoptosis up to 72 h, neither IL-5 nor FBS alone did inhibit apoptosis to the significant levels observed with the IL-5 and FBS combination. Moreover, there was no difference in apoptosis between eosinophils obtained from allergic and allergic asthmatics and eosinophils obtained from non-allergic nonasthmatic individuals. This suggested to us that the increased survival rate of eosinophils in the airway tissue of asthmatics might be due to extrinsic factors (IL-5 and other cytokines hypersecretion) rather than intrinsic factors (e.g., up-regulation of antiapoptosis proteins and down-regulation of proapoptosis proteins). Alternatively, the effect of IL-5 or other eosinophil growth factors (IL-3 and GM-CSF) could be transient. However, this does not rule out the possibility that there might be some intrinsic and genetic differences between eosinophils from normal, allergic, and allergic asthmatic subjects.

We have shown that eosinophils undergoing apoptosis possess significant levels of caspase-3, caspase-8, and caspase-9 activity. In fact, there was a 12-fold increase in caspase-3 activity, and a 3-fold increase in caspase-8 and caspase-9 activities in eosinophils incubated in serum-deficient conditions for 24 h. Interestingly, when eosinophils were incubated with FBS and IL-5 for 24 h, caspase activity remained relatively low and comparable to the levels observed in freshly isolated eosinophils. These findings indicate a possible involvement of caspase-3, caspase-8, and caspase-9 in eosinophil apoptosis, and that IL-5 and FBS might protect eosinophils from apoptosis probably by suppressing caspase activation. However, the exact role of caspase-3, caspase-8, and caspase-9 in modulating apoptosis in our model needs to be further investigated.

Many aspects of eosinophil apoptosis mechanisms have yet to be identified especially the role of novel cell surface molecules in the regulation of eosinophil survival and apoptosis. Our microarrays observation of CD30 expression by eosinophils was confirmed by flow cytometry using FITC-conjugated anti-CD30 Abs.

**FIGURE 7.** Effect of CD30 Abs on serum-deprived human blood eosinophil apoptosis in vitro. Purified eosinophils were resuspended in RPMI 1640 medium supplemented with only 1% FBS and layered over the well-bound Abs or control IgG in 24-well plates. Apoptosis was determined by flow cytometry after 12 h (a), and 24 h (b) as described in Materials and Methods. Data represent mean ± SEM of n = 3; *, p < 0.05 and **, p < 0.01 vs the no Ab control group.
In this study we report for the first time the correlation between human blood eosinophils apoptosis and expression of CD30 molecule. The surface CD30 expression was significantly up-regulated when eosinophils were incubated for 24 h, especially under serum-deficient conditions (Figs. 5 and 6). We also performed qRT-PCR experiments to determine the levels of CD30 mRNA expression by eosinophils under different conditions. CD30 mRNA expression increased in eosinophils incubated with 1% FBS for 12 and 24 h as compared with the freshly isolated eosinophils (Fig. 4). To our surprise, CD30 mRNA expression in eosinophils incubated with 10% FBS and IL-5 did not increase at 12 h, rather it slightly, yet insignificantly, decreased at 24 h (Fig. 4, b and c). In comparison, CD30 surface expression in the presence of 10% FBS and 15 ng/ml IL-5 increased over time (Figs. 5 and 6b). Interestingly, the qRT-PCR data did not correlate with the semiquantitative RT-PCR results (Fig. 4a) in which CD30 mRNA expression in eosinophils incubated with 10% FBS and IL-5 appears to have increased, and not decreased, after 24 h of incubation. This could be explained by

**FIGURE 8.** Effect of CD30 Abs on serum-sufficient human blood eosinophil apoptosis in vitro. Purified eosinophils were resuspended in RPMI 1640 medium supplemented with 10% FBS and 15 ng/ml IL-5 then layered over the well-bound Abs or control IgG in 24-well plates. Apoptosis was determined by flow cytometry after 12 h (a) and 24 h (b) as described in Materials and Methods. Data represent mean ± SEM of n = 3; *, p < 0.05 and **, p < 0.01 vs the no Ab control group.
the fact that semiquantitative RT-PCR is much more prone to variations in reverse transcription, PCR amplification efficiencies, and normalization errors than qRT-PCR (37, 38). Moreover, difference in endogenous genes used for normalization (RPL13A vs GAPDH) could be responsible as well. Many studies have revealed that the expression levels of these genes can vary and are directly influenced by the experimental conditions (39, 40). Studies are currently underway to determine the most reliable and efficient housekeeping gene to use in our system.

It has also been reported that eosinophils from normal subjects and subjects with IgE-mediated allergic diseases do express some surface markers differently, like CD137 that is expressed only on the surface of eosinophils isolated from allergic individuals (41). Therefore, we obtained eosinophils from normal subjects and determined by flow cytometry that they do express surface CD30 in a similar fashion to the eosinophils isolated from allergic individuals (data not shown).

The observation that the increase in CD30 expression coincides with the elevated levels of eosinophil apoptosis in vitro suggested a possible engagement of CD30 molecules in the regulation of human blood eosinophil apoptosis. Unlike most members of the TNFR family, CD30 lacks the death domain in its cytoplasmic portion. However, they contain TNFR-associated factor (TRAF) domains that have been shown to interact with TRAF-1, TRAF-2, TRAF-3, and TRAF-5 through which it mediates NF-kB activation. Interestingly, CD30 molecule has been linked to apoptosis and cell arrest in many cellular systems. Gruss et al. (30) were among the first to demonstrate the pleiotropic biological effects of CD30 activation in different CD30+ lymphoma cells, like enhancing proliferation of T cell-like but not B cell-like Hodgkin’s disease-derived cell line, and inducing cytolytic cell death of large-cell anaplastic lymphoma cell lines. Later, Lee et al. (42) demonstrated that multimerization of CD30 cytoplasmic domains induces apoptosis of T cell hybridomas when it accompanied TCR activation. Studies using CD30−/− mice suggested a possible role for CD30 in negative selection in the thymus. More recently, this hypothesis gained more support when Chiarle et al. (43) showed that in transgenic mice overexpressing CD30 in T lymphocytes, CD30 transgenic thymocytes were induced to undergo apoptosis upon CD30 cross-linking and CD3 activation. The same study also revealed that CD30 cross-linking alone also enhanced spontaneous apoptosis. Mir et al. (31) also demonstrated that activation of CD30 leads to the induction of apoptotic death of anaplastic large cell lymphoma cells. One possible mechanism by which CD30 activation induces apoptosis is the recruiting and degradation TRAFs, thus rendering the cell sensitive for receptor-mediated apoptosis (44). The same study also revealed that CD30 cross-linking alone also enhanced spontaneous apoptosis. Taken altogether, these observations imply a potential physiological role for CD30 as an apoptotic costimulatory molecule.

Based on our observations that levels of CD30 expression correlate with those of apoptosis in our system, we pursued an answer to the question of whether CD30 expression in eosinophils is a cause or consequence of apoptosis. Increased CD30 expression before apoptosis would indicate a direct role for CD30 in the induction of apoptosis. Our time-course experiments unveiled that both CD30 expression and apoptosis increased in a time-dependent manner. The addition of IL-5 and FBS, though reduced both CD30 expression and apoptosis, did not however alter the time-dependent pattern. Incubating eosinophils with CD30 agonistic Abs Ber-H8 and HeFi-1 revealed that CD30 activation resulted in significant increase in apoptosis as compared with incubation with nonagonistic and control Ab. The effect of CD30 activation on eosinophil apoptosis was readily observed at 12 h.

The last question we attempted to address is whether IL-5 and FBS are capable of blocking this CD30-induced apoptosis in eosinophils. To our surprise, IL-5 and FBS were ineffective. CD30 activation with the agonistic Abs Ber-H8 and HeFi-1 Abs induced very significant increase in eosinophil apoptosis at 12 and 24 h postincubation. These observations implied that CD30 activation might be inducing eosinophil apoptosis via separate and independent intracellular pathways that are not sensitive to survival effect of IL-5. However, eosinophils apoptosis induced by CD30 activation might involve separate and independent intracellular signals of the intracellular survival signals mediated by IL-5, or perhaps overwhelming the IL-5-mediated survival signals. It is also possible that CD30 activation is interfering with the effect of FBS and IL-5 on CD30 transcription and translation, therefore allowing more CD30 molecules to be expressed on cell surface, resulting in more CD30 activation and apoptosis. This hypothesis is supported by the fact that CD30 activation results in the significant induction of apoptosis in eosinophils in the presence of IL-5 and FBS, despite the fact that these cells display relatively low levels of CD30 surface expression. Thus, it appears that the amount of CD30 expression correlates with apoptosis only in spontaneous apoptosis. However, it is the activation of CD30, and not the total expression of CD30 on the cell surface, which is important for the induction of apoptosis even in the presence of IL-5 and FBS. Experiments are underway to analyze the intracellular molecules and pathways that CD30 activation might be using to induce eosinophil apoptosis. Some proteins of interest are the TRAF-interacting proteins that have been identified and shown to play a critical role in modulating the signals mediated through TRAF activation. Cellular inhibitors of apoptosis protein-1 and protein-2, along with the TRAF-associated NF-kB-activator TANK, are among the most important (45, 46). To date, there are no reports about any of the TRAF proteins or any other TRAF-interacting proteins in human blood eosinophils. Therefore it is warranted to investigate the expression and unveil the role of TRAF proteins and TRAF-interacting proteins in the apoptosis of human blood eosinophils.

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