CD40 Expression by Human Peripheral Blood Eosinophils

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Abstract

In this study, we have investigated CD40 expression in human peripheral blood eosinophils and in human chronically inflamed nasal tissues, i.e., nasal polyps. We show by both reverse transcriptase–PCR and Northern blot analysis that eosinophils from allergic subjects express human CD40 mRNA. We also show that constitutive CD40 mRNA expression in eosinophils could be upregulated by exposure to IgA immune complexes and downregulated by IL-10 and the synthetic steroid budesonide. In addition, we demonstrate that eosinophils express CD40 protein by flow cytometry. Such expression is biologically functional as cross-linking CD40 with CD40 mAbs enhances eosinophil survival in a dose-dependent fashion; in addition, CD40 ligation stimulates eosinophils to release GM-CSF. CD40-mediated eosinophil survival was largely inhibited by an anti–GM-CSF neutralizing antibody suggesting GM-CSF involvement in the survival enhancing mechanism. CD40 mRNA was also detected in total RNA extracted from nasal polyp tissues but not in RNA isolated from normal nasal mucosa (inferior turbinate); by immunohistochemistry, we were able to detect immunoreactive CD40 protein in a variety of cell types in the polyp stroma, but primarily in eosinophils. These observations suggest previously unforeseen interactions between eosinophils and cells expressing the CD40 ligand and, thus, novel pathways by which eosinophils may contribute to the regulation of airway inflammation. (*J. Clin. Invest.* 1996. 97:1761–1766.) Key words: GM-CSF • nasal polyp • CD40L (gp39) • IL-10 • glucocorticoid

Introduction

CD40 is a 45–50 kD transmembrane glycoprotein originally identified in B cells, some B cell malignancies and carcinoma cell lines (1, 2). More recently, CD40 expression has also been recognized in other cell types including follicular dendritic cells (3), thymic epithelial cells (4), monocytes (5), fibroblasts (6, 7), and endothelial cells (8). CD40 is a member of the nerve growth factor/TNF receptor superfamily (9–11). CD40 signaling can be initiated by cross-linking CD40 with anti-CD40 mAb or by interaction with CD40 ligand (CD40L), now referred to as gp39, which is expressed by $CD4^+$ T cells (12–14) and, as recently shown, by human mast cells and basophils (15, 16). The importance of CD40–CD40L interactions in vivo has been recently demonstrated in autoimmune diseases such as collagen-induced arthritis (17), acute and chronic graft-versus-host disease (18), and lupus nephritis (19) where treatment of experimental animals with anti-gp39 antibodies markedly prevented disease expression. In addition, this receptor/ligand interaction is, in the presence of IL-4 as a coactivation signal, thought to play a key role in the switching of B cells to an IgE phenotype, hence implicating CD40 in allergy. However, there has been a lack of direct evidence to date showing the involvement of the CD40 signaling system in allergic inflammatory diseases. The primary objective of this study was to determine whether human peripheral blood eosinophils, the most prevalent inflammatory cell type in such type of inflammation, express CD40 and, secondarily to investigate some of the functional consequences upon CD40 signaling. In addition, we examined CD40 expression in human inflamed nasal polyp tissues, which, in allergic subjects, are characterized by marked eosinophil infiltration.

Methods

Reagents. Recombinant human (rh)[1] GM-CSF, rhIL-10, specific mouse monoclonal neutralizing antibodies against human GM-CSF, IL-3, IL-5 and mouse anti–human CD40 mAb (5C3) directly conjugated FITC were purchased from R & D Systems, Inc. (Minneapolis, MN). Mouse anti–human CD40 mAb (5C3) was purchased from PharMingen (San Diego, CA). Control mouse myeloma protein MOPC-21 (IgG_1), human serum IgA, anti–human IgA mAb, PMA, and calcium ionophore (A23187) were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse IgG_1 directly conjugated FITC was purchased from Becton Dickinson & Co. (Mountain View, CA). Micromagnetic beads bound to anti-CD16 mAb and magnetic-activated cell sorter (MACS) columns were supplied by Miltenyi Biotec Gmbh (Gergisch-Gladbach, Germany). Budesonide was provided from Astra Pharma Inc. (Mississauga, Canada).

Purification and culture of human peripheral eosinophils. A volume of 100 ml of blood was obtained from atopic (skin test positive) subjects with mild symptoms of allergic rhinitis, asthma, or both. Eosinophils were purified by negative-immunomagnetic selection as previ-

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1. *Abbreviations used in this paper:* rh, recombinant human; RT, reverse transcriptase.

ously described (20). This procedure consistently resulted in a highly purified eosinophil population (> 99%). These eosinophils (> 99% viable by trypan blue exclusion) were cultured in RPMI supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin with or without the addition of cytokines and specific mAbs.

Flow cytometry. Freshly isolated eosinophils were resuspended at 4°C in HBSS with 1% BSA and 0.1% NaN_3 (FACS® buffer) at 2×10^5 cells per 80 μl. 80 μl of cell suspension was incubated with 10 μl of anti-CD40 mAb directly conjugated FITC or isotype-matched control Ab at 4°C in the dark for 30 min. Cells were washed with FACS buffer once, collected by centrifugation at 250 *g* and fixed with 0.5% paraformaldehyde in FACS buffer before scanning. Flow cytometric analysis was performed using a FACScan (Becton Dickinson).

Reverse transcriptase (RT)-PCR and Southern blot analysis. Oligonucleotides for PCR of human CD40 were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada. The sequence of antisense primer for human CD40 was 5′-ATC CTG GGG ACC ACA GAC AAC ATC AGT-3′, which was complementary to nucleotides 591 through 617, and the sequence of sense primer was 5′-ATG GTT CGT CTG CCT CTG CAG TGC-3′, which was homologous to nucleotides 1 through 24 of the human CD40 cDNA sequence (9). Amplification with these primers was expected to yield a 570-bp product. The sequence of antisense and sense primers for β-actin was 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′ and 5′-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3′. Total RNA was extracted from freshly isolated eosinophils or eosinophils cultured with or without the potent synthetic steroid budesonide (10^{-6} M) or rhIL-10 (10 ng/ml) for 3 h using the RNAzol method (Biotecx Laboratories, Houston, TX). RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) according to the manufacturer's protocol. RNA samples (2 μg of total RNA), 40 U of rRNasin (recombinant ribonuclease inhibitor; Promega Corp.), 0.5 μg of random primer (Promega Corp.), 10 mM of each deoxynucleoside triphosphate, and 20 U of reverse transcriptase were incubated in a total of 25 μl of reaction mixture containing the enzyme buffer as supplied by the manufacturer. The reaction mixture was incubated for 1 h at 42°C. The reverse-transcripted products were then amplified with Taq DNA polymerase (Promega) following the manufacturer's protocol. 100 μl of PCR mixture consisted of the PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100; Promega, pH 9.0), 1.25 mM of each deoxynucleoside triphosphates, 5 μl of the reverse-transcribed product, 150 pmol of both antisense and sense primers, and 2.5 U of Taq DNA polymerase, with 50 μl of mineral oil (Sigma Chemical Co.) layered on the surface of the reaction. 40 cycles of PCR were performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 2 min for enzymatic primer extension at 72°C. Specific PCR product for human CD40 was determined by Southern blot and hybridization as previously described (21). After Southern transfer and hybridization, the filter was exposed to a Kodak x-ray film at −70°C for 20 min.

Northern blot analysis. Total RNA was extracted from freshly isolated eosinophils, eosinophils incubated with IgA-immune complexes (15 μg/ml of human IgA and 20 μg/ml of anti–human IgA) for 18 h, and nasal polyp tissues by RNAzol or acid guanidine thiocyanate-phenol-chloroform extraction (22). Approximately 2 μg of total RNA could be extracted from one million eosinophils. Total RNA was separated on a 1.2% agarose gel and transferred onto Duralon UV membrane (Stratagene Corp., La Jolla, CA) and hybridized with human CD40 probe that was labeled using T7 Quick Prime™ Kit (Pharmacia, Uppsala, Sweden) with [α-^{32}P]dCTP.

Eosinophil survival. Eosinophil survival was assessed as previously described (20). Briefly, eosinophils (2×10^5/well in 0.2 ml RPMI) were cultured in 96-well microtiter plates (Corning, New York, NY). Cells were removed from each well after gentle pipetting and viability was assessed at day 4 by trypan blue exclusion using a hemocytometer.

Immunohistochemistry. Nasal polyp tissues were cut into small pieces, fixed in periodate-L-lysin-paraformaldehyde, embedded with OCT compound (Miles Laboratories Inc., Naperville, IL) in liquid nitrogen after washing with sucrose/PBS and stored at −70°C. Sections 6-μm thick were thawed and rinsed in TBS (0.05 M Tris, 0.15 M NaCl, pH 7.6). To block nonspecific reactions, slides were preincubated for 30 min at room temperature in 10% heat-inactivated normal rabbit serum in TBS. Slides were then subjected to staining for CD40 protein by using anti-CD40 mAb (5C3; PharMingen) as the first Ab. 10 μl/ml of the first mouse Ab, either anti-CD40 mAb or control mAb (IgG_1), were used to stain cells. Slides were incubated overnight at 4°C. Then, slides were washed in TBS and incubated with anti–mouse Ig (Dako Corp., Carpinteria, CA) for 60 min at room temperature and further incubated with mouse alkaline phosphatase anti-alkaline phosphatase (APAAP) complex (Dako Corp.) for 60 min at room temperature after washing with TBS. Alkaline phosphatase substrate in the presence of levamisole to block endogenous alkaline phosphatase was used for signal detection (Fast Red Substrate System; Dako). The slides were stained with 1 μg/ml FITC (Sigma) for 10 min to identify eosinophils (23), counterstained with Mayer's hematoxylin solution (Sigma) for 2 min and mounted with GVA-MOUNT (Zymed Laboratories Inc., San Francisco, CA).

Cytokine assay. Eosinophils (5×10^5/well in 0.5 ml of RPMI) were cultured in 24-well plates (Corning), and supernatants were collected at 24 h and stored at −20°C until assayed. GM-CSF, IL-3, and IL-5 were measured using commercially available ELISA kits (Quantikine; R & D Systems). The sensitivity of detection was 1.5 pg/ml, 7.4 pg/ml, 1.0 pg/ml for GM-CSF, IL-3, and IL-5, respectively.

Data analysis. Data were expressed as mean±SEM. Wherever suitable, interpretation of results was done by ANOVA. The difference was considered statistically significant when $P < 0.05$.

Results and Discussion

CD40 mRNA expression in human peripheral blood eosinophils. It has been reported that CD40 is constitutively expressed in several structural cell types in addition to B cells (4, 9). To investigate whether human peripheral blood eosinophils express CD40, we first examined mRNA expression using RT-PCR. As shown in Fig. 1 *A*, the correct 570-bp sized RT-PCR product of mRNA was detected in freshly isolated eosinophil preparations (lanes *1* and *2*). As a positive control (lane *3*), we used PBMCs that were stimulated with PMA and calcium ionophore (A23187). The specificity of this product was shown by hybridization with human CD40 cDNA. Despite the very high purity of the eosinophil preparations used in these experiments, we wished to confirm CD40 mRNA expression in human peripheral blood eosinophils by an independent method and, also, to determine whether this message could be induced. To this end, total RNA of eosinophil preparations from eight different allergic subjects was isolated and pooled. As shown in Fig. 1 *B*, the expected 1.3-kb sized CD40 mRNA was clearly detectable in total RNA from these eosinophils by Northern blot analysis (lane *1*). Since immune-complexes, particularly IgA, have been shown to be a most potent stimulus capable of upregulating IL-5 synthesis as well as eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP) release by eosinophils (24–26), we used this approach to examine CD40 mRNA induction. As seen in lane *2* (Fig. 1 *B*), incubation of eosinophils with IgA immune-complexes for 18 h resulted in upregulation of the CD40 message. Again, CD40 message in mononuclear cells served as positive control and size marker (lane *3*).

Glucocorticoids have been shown to have a broad spec-

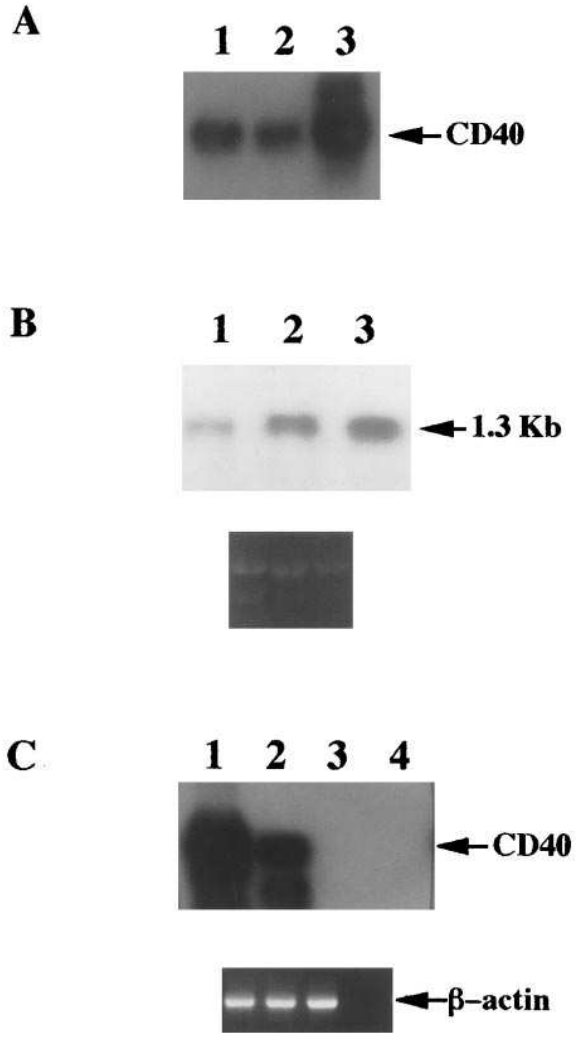

Figure 1. CD40 mRNA expression by human peripheral blood eosinophils. (*A*) Southern blot analysis of RT-PCR products from RNA samples. Total RNA extracted from freshly isolated peripheral blood eosinophils from two different subjects (purity > 99.5%) (lanes *1* and *2*) or mononuclear cells cultured with PMA (2 ng/ml) and A23187 (10^{-7} M) for 18 h (lane *3*) was reverse transcribed and amplified with antisense and sense primers. The RT-PCR products were blotted and hybridized with ^{32}P-labeled CD40 cDNA probe. (*B*) Northern blot analysis for CD40. Total RNA from freshly isolated eosinophils (lane *1*) or eosinophils cultured with IgA immune-complexes for 18 h (lane *2*), which were prepared from eight different allergic subjects and pooled, or freshly isolated mononuclear cells (lane *3*) was blotted and hybridized with ^{32}P-labeled human CD40 cDNA probe. Ethidium bromide staining to confirm equivalent loading of total RNA is also shown. (*C*) Regulation of CD40 mRNA expression on eosinophils by glucocorticoid and rhIL-10. Freshly isolated eosinophils were incubated for 3 h with rhIL-10 (10 ng/ml) or budesonide (10^{-6} M), and Southern blot analysis was performed on the RT-PCR products from isolated mRNA (lane *1*: RPMI, lane *2*: rhIL-10, lane *3*: budesonide, lane *4*: negative control [water]). The result of RT-PCR for β-actin is shown to confirm that equal amounts of RNA were used.

trum of antiinflammatory and immune downregulatory activities and have an established role in the treatment of allergic diseases. Similarly, IL-10 has a number of well documented anti-inflammatory effects and, with respect to eosinophils, we have previously shown that IL-10 inhibits cytokine release from stimulated human peripheral blood eosinophils in vitro (20). Thus, we felt it of interest to determine whether steroid and/or IL-10 could downregulate CD40 mRNA expression in these cells. As shown in Fig. 1 *C*, unstimulated eosinophils cultured for 3 h in medium alone constitutively express CD40 mRNA (lane *1*). Exposure of eosinophils for 3 h to rhIL-10 (10 ng/ml) (lane *2*) or to the potent synthetic steroid budesonide (10^{-6} M) (lane *3*) clearly, and in the case of budesonide remarkably, inhibited CD40 mRNA expression. These data show that CD40 expression in human blood eosinophils can be downregulated by antiinflammatory agents.

CD40 protein expression and effect of CD40 cross-linking on peripheral blood eosinophils. Having shown CD40 mRNA expression in human peripheral blood eosinophils, we investigated CD40 protein expression by flow cytometry. FACS analysis was carried out in 14 allergic subjects. CD40-positive eosinophils were detected in 12 donors. In these, ~ 40% of freshly isolated peripheral blood eosinophils were positive (Fig. 2). Since it has been reported that cross-linking of CD40 by anti-CD40 mAb prevents apoptosis of germinal center B cells (27, 28), we next asked whether CD40 cross-linking would regulate eosinophil survival. To this end, human peripheral blood eosinophils were incubated with several concentrations of anti-CD40 mAb or IgG_1 as a negative control, both of which are Fc-containing antibodies. As shown in Fig. 3 *A*, cross-linking of CD40 by mAb significantly prolonged eosinophil survival in a dose-dependent fashion, 54.1±5.8% with 1 μg/ml and 72.3±5.2% with 10 μg/ml ($n = 6$) in contrast to the minimal effect elicited by control IgG_1.

We and others have previously shown that GM-CSF has a potent effect on eosinophil survival (29–31). Thus, we investigated whether this cytokine was involved in CD40-induced eosinophil survival. To test this, freshly isolated eosinophils were cultured in the presence of anti-CD40 mAb, and the content of GM-CSF in the 24 h supernatant was measured by ELISA. We were able to detect 6.8±1.7 pg/ml ($n = 4$) of GM-CSF in these eosinophil supernatants, and no GM-CSF could be detected in the supernatant of eosinophils cultured with equivalent amounts of IgG_1. Under the same culture conditions, we found that rhGM-CSF (5–10 pg/ml) enhanced eo-

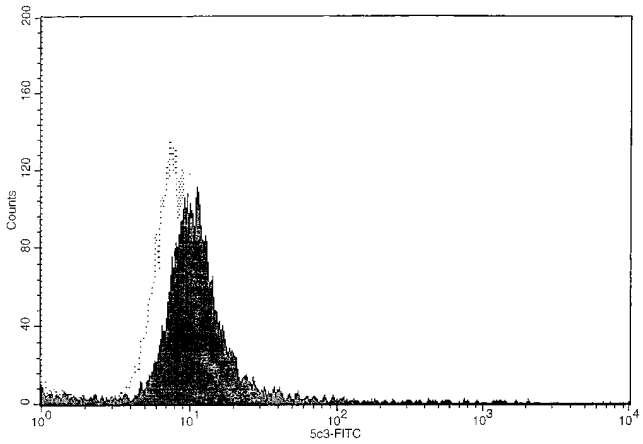

Figure 2. Flow cytometric detection of CD40 expression in freshly isolated peripheral blood eosinophils. Eosinophils stained positively with FITC conjugated anti-CD40 mAb (*filled*) compared to FITC conjugated IgG_1 control mAb (*broken line*).

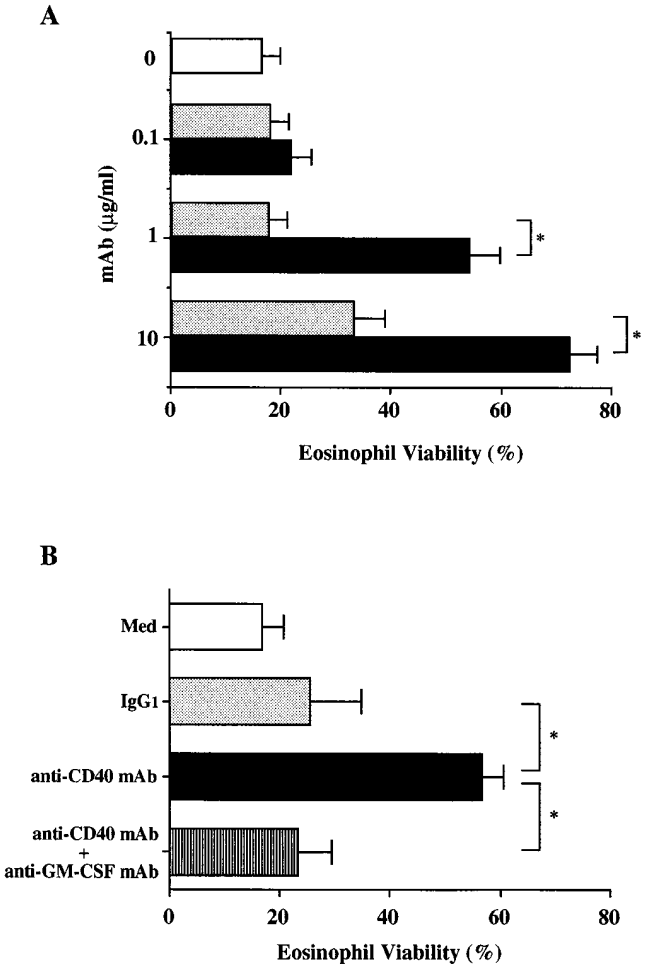

Figure 3. Effect of CD40 cross-linking on eosinophil survival. (*A*) Freshly isolated eosinophils (2×10^5) were cultured with increasing concentrations of an anti-CD40 mAb (*solid bars*) or a control mAb (IgG_1) (*dotted bars*), and eosinophil survival was assessed at day 4 by trypan blue dye exclusion. (*B*) Freshly isolated eosinophils were cultured in medium alone (*empty bar*), or with a control IgG_1 mAb (10 μg/ml) (*dotted bar*), or with an anti-CD40 mAb (10 μg/ml) in the absence (*solid bar*) or presence (*hatched bar*) of an anti–GM-CSF neutralizing mAb (0.5 μg), and eosinophil survival assessed. Results are expressed as mean±SEM from six (*A*) or four (*B*) independent experiments. *Statistically significant difference ($P < 0.05$).

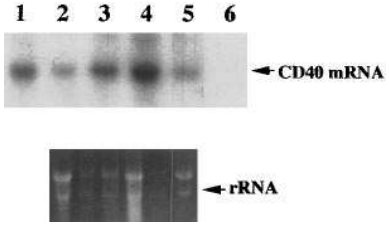

Figure 4. Northern blot analysis for CD40 gene expression in nasal polyp tissues. Total RNA from five different nasal polyp tissues (lanes *1–5*) and one normal nasal mucosa (lane *6*) was blotted and hybridized with ^{32}P-labeled CD40 cDNA. Ethidium bromide staining shows the amount of total RNA loaded to the gel (lanes *1*, *4*, *6*: 20 μg of total RNA; lanes *2*, *3*, *5*: 10 μg of total RNA).

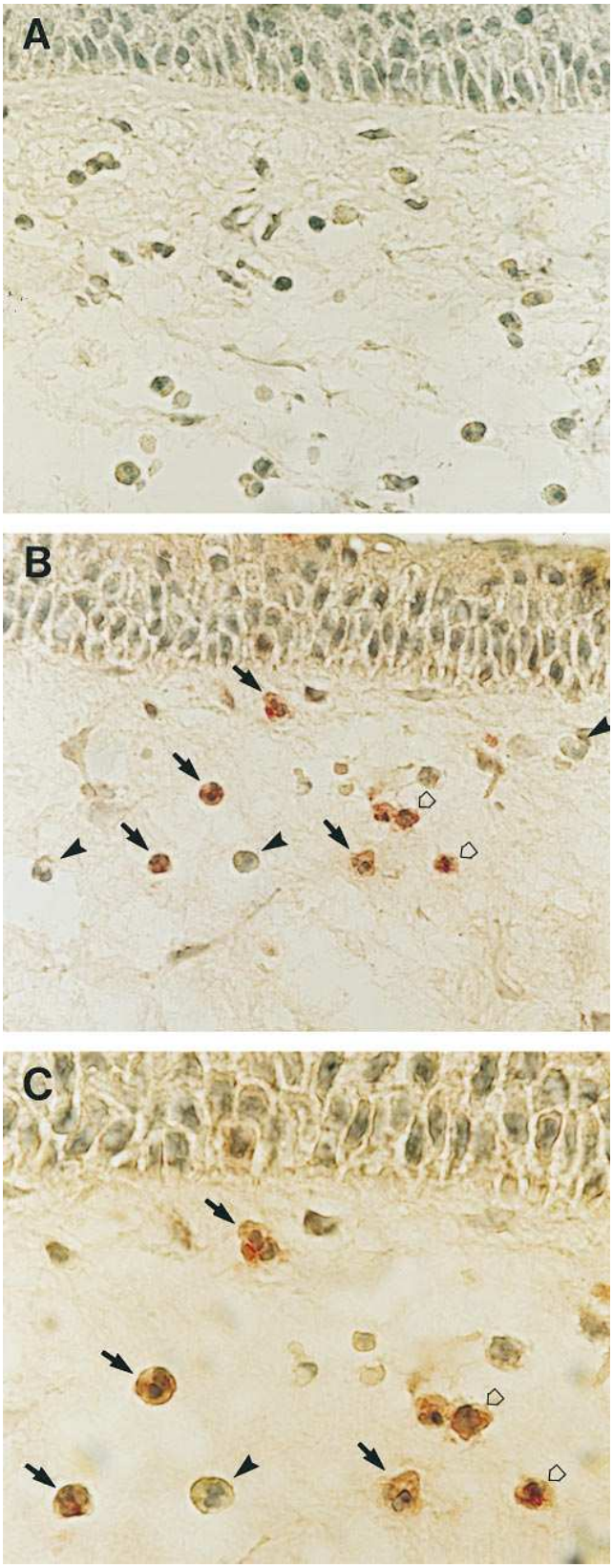

Figure 5. Immunohistochemical detection of CD40 protein in nasal polyp tissue. Nasal polyp tissue section (6 μm) stained with a control mAb (IgG_1) at 10 μg/ml (*A*, ×400) and an anti-CD40 mAb at the same concentration (*B*, ×400; *C*, ×640). Black arrows indicate CD40-positive eosinophils, arrow heads indicate CD40-negative eosinophils, and open arrows indicate CD40-positive mononuclear cells.

sinophil survival to levels between 50–75%, i.e, similar to those induced by exposure to 10 μg/ml of anti-CD40 mAb. There was no detectable IL-3 and IL-5 in the supernatant of anti-CD40–stimulated eosinophils as measured by ELISA. Furthermore, the enhanced eosinophil survival induced by CD40 cross-linking was reduced to control levels by concurrent incubation with a specific anti–GM-CSF mAb (Fig. 3 *B*) but not with either anti–IL-3 mAb or anti–IL-5 mAb (data not shown). Together, these results indicate that human blood eosinophils express biologically functional CD40 and that activation of this molecule increases eosinophil survival via induction of GM-CSF release.

CD40 expression in vivo. To our knowledge, there is no evidence to date of CD40 expression in human inflamed airways tissues. To investigate this, we set out to examine CD40 expression in nasal polyps, which may be viewed as a paradigm of chronic mucosal inflammation; they are often associated with asthma and, in allergic subjects, are characterized by marked infiltration of activated eosinophils. By Northern blot analysis, a specific message for CD40 was detected in total RNA from several nasal polyp tissues (Fig. 4, lanes *1–5*). In contrast, only a very faint signal was observed in normal control nasal mucosa (Fig. 4, lane *6*). Examination of nasal polyp tissues by a double staining with immunohistochemistry for CD40 and FITC for eosinophils showed that the majority of cells in the polyp stroma localizing CD40 immunoreactivity were eosinophils (*black arrows*), although some mononuclear cells (*open arrows*) also stained positive (Fig. 5 *A* and *C*). No immunoreactivity was observed in tissues stained with the control IgG_1 antibody (Fig. 5 *B*). These data demonstrate expression of immunoreactive CD40 by eosinophils in vivo in allergic inflammation.

In summary, we have shown that peripheral blood eosinophils from allergic subjects express CD40 mRNA and protein and that cross-linking of CD40 with anti-CD40 mAb results in enhanced survival of eosinophils, which appears to be mediated by GM-CSF. We also show CD40 mRNA expression in chronically inflamed human airway tissues and provide evidence of CD40 immunoreactivity on eosinophils infiltrating these tissues. These findings suggest previously unrecognized interactions between eosinophils and CD40 ligand-expressing cells thought to be important in allergic inflammation such as T lymphocytes (12–14) and mast cells (16). A recent report indicating that eosinophils themselves can express CD40 ligand (32) suggests that CD40–CD40L interactions may be involved in autocrine/paracrine modes of stimulation in eosinophils as it has been suggested for B cells (33). In addition, we have recently shown that human eosinophils can synthesize IL-4 and that, in nasal polyp tissues, these cells are an important source of immunoreactive IL-4 (23). Thus, the ability of eosinophils to make IL-4 and to express CD40 suggests novel roles for eosinophils in mucosal immune-inflammatory responses. The relevance of the CD40 system in allergic inflammation in vivo remains to be determined. However, the fact that cross-linking of CD40 on thymic epithelial cells, monocytes and, as we show here, eosinophils results in the synthesis and release of a number of proinflammatory cytokines does implicate CD40 in the regulation of allergic inflammation. We speculate that interference with the CD40 signaling system, whether by inhibition or by blockade, may have a profound effect in multiple cell-to-cell interactions in the tissue and, hence, in the regulation of inflammatory reactions.

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