Human Eosinophils Express Functional CD30 Ligand and Stimulate Proliferation of a Hodgkin’s Disease Cell Line

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The presence of a prominent tissue eosinophilia represents a typical histopathologic hallmark of Hodgkin’s disease (HD). To evaluate the putative role of eosinophils in tumor cell regulation in HD, we have analyzed these cells for the functional expression of CD30 ligand (CD30L), a surface molecule able to transduce CD30-mediated proliferation signals on Hodgkin’s (H) and Reed-Sternberg (RS) cells. The results demonstrate that circulating and tissue eosinophils from normal donors and patients with HD or hypereosinophilic syndrome (HES), display CD30L mRNA and express CD30L protein, as shown by immunostaining with a specific monoclonal antibody (M80) and with a biotinylated soluble CD30-Fc fusion protein. The surface density of CD30L on eosinophils from HD and HES patients was remarkably higher compared with healthy donors, probably reflecting a cytokine-mediated upregulation in these pathologic conditions. Accordingly, we provide evidence that cytokines regulating eosinophil proliferation and activation, i.e., interleukin-5 (IL-5), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF), are able to enhance the cellular density of CD30L on purified eosinophils from normal subjects. Finally, we show that native CD30L on human eosinophils is a functionally active surface structure able to transduce proliferative signals on CD30+ target cells, including cultured H-RS cells. Our data suggest that eosinophils may not merely represent innocent bystanders, but rather act as important elements in the pathology of HD by contributing to the deregulated network of CD30/CD30L-mediated interactive signals between H-RS cells and surrounding reactive cells.

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via a CD30-mediated intracellular signalling pathway, also causing cellular activation with enhanced cytokine secretion (eg, IL-6, TNF, LT-α) and surface antigens expression (eg, CD54, CD80, CD86).11,12,27 CD30 and CD30L might therefore represent a molecular pair critically involved in the control of tumor cell growth and activation in HD.11,18,22-27

To clarify the pathobiologic significance of eosinophil infiltration in HD, we investigated whether circulating or tissue eosinophils from normal individuals or HD patients could express functionally active CD30L and assessed the ability of purified eosinophils to stimulate proliferation of cultured H-RS through a CD30-dependent mechanism.

**MATERIALS AND METHODS**

**Eosinophil isolation and purification.** PB buffy coats were obtained from consenting healthy donors (n = 10), patients with HD (n = 5), and patients with hypereosinophilic syndrome (HES)18-20 (n = 4). In addition, peripheral blood mononuclear cells were collected from three patients with HD who were studied.20,28 To isolate eosinophils a two-step procedure was used which combines density gradient centrifugation with removal of contaminating neutrophils by negative immunomagnetic selection, as described.29,30 Briefly, after 2.25% dextran sedimentation cells were washed and centrifuged through isopaque (osmolality of 285 to 294 mOsm/kg) discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradients, with densities ranging from 1.077 g/mL to 1.105 g/mL.31 as measured with a pycnometer at 20°C. Interfaces at a specific gravity of 1.090, 1.095, and 1.105 g/mL were collected and depleted of contaminating neutrophils by incubation with the anti-CD16 monoclonal antibody (MoAb) CLB/FcR gran 1 (Miltenyi Biotec Inc, Celbio, Milan, Italy) followed by sheep anti-mouse IgG-coated immunomagnetic beads (Dynabeads, Dynal, Norway). Eosinophil preparations (95% to 98% pure, as assessed by May-Grünwald Giemsa staining) were either fixed in 0.5% paraformaldehyde or resuspended in Iscove's modified Dulbecco medium (IMDM; Gibco, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS; Gibco) and used for immunofluorescence studies, RNA preparation, in vitro cultures, and functional assays.

**MoAbs and flow cytometry.** The anti-CD30L MoAb M80 (mouse IgG2b isotype) was developed and characterized as described.31 Indirect immunofluorescence was performed by sequentially incubating cells with saturating concentrations of the anti-CD30L MoAb M80 (10 μg/mL) and fluorescein isothiocyanate (FITC)-conjugated Fab’ fragments of goat anti-mouse IgG (H + L) (Technogenetics, Milan, Italy), as described.31 Non-specific binding of MoAbs was assessed by labeling cells with isotype-matched control mouse IgG (Jackson Immunoresearch Laboratories, Inc, West Grove, PA). Viable, antibody-labeled cells were identified according to their forward and right angle scattering, electronically gated and analyzed for surface fluorescence on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Fluorescence values were also converted into the number of molecules of equivalent soluble fluorochrome per cell (MESF) by comparing the results of anti-CD30L staining with calibrated fluorescence reference standard microbeads (Flow Cytometry Standards Corporation, Inc, Research Triangle Park, NC), which were run in parallel with the samples in each experiment, as described.31 Fluorescence intensity achieved with an isotype-matched control antibody was subtracted to calculate the net MESF ± SEM values for the anti-CD30L MoAb M80. The statistical significance of differences in MESF values was calculated by the Student’s t-test. Surface expression of CD30L was also assayed by incubating cells with 5 μg/mL of biotinylated CD30-Fc fusion protein, obtained by fusing the extracellular domains of CD30 to the Fc region of human IgG1 (kindly provided by Immunex Research and Development Corp, Seattle, WA), and biotinylated control IgG1 for 30 min at 4°C, as previously described.24,25 Cells were then washed, incubated with streptavidin-phycocerythrin (Becton Dickinson), and analyzed by flow cytometry.

**Immunohistochemistry.** Reactive (n = 9), angioimmunoblastic lymphadenopathy (AILD)-involved (n = 3), and HD-involved (n = 7) lymph node tissues were fixed in Bouin solution or neutral buffered formalin.

Anti-CD30L MoAb M80 was applied to deparaffinized tissue sections from all cases included in the study. Immunohistochemistry was performed with the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method as described.20,29 Cytospin smears of purified eosinophil preparations were fixed in neutral buffered formalin at room temperature for 10 minutes and then immunostained with the anti-CD30L MoAb M80 by the APAAP method.20,29,31

**RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA (1 μg), extracted by the guanidium thiocyanate method,20,32 was reverse-transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Promega Co, Madison, WI) in a 20-μL reaction mix containing hexadecyltrimethylammonium chloride random primers (0.4 μg) for 1.5 hours at 42°C. Five microliters of the same cDNA preparations was amplified in a 50-μL vol of final reaction mix in a Perkin Elmer 9600 thermal cycler (Perkin Elmer Cetus, Emeryville, CA), with 25 pmol/L of primers specific for CD30L (sense, 5'-CCC TCC TGG AGA CAC AGC-3', region 1980-1960; antisense, 5'-CTT GAA GGC CAA GAG AAA CTG-3', region 1841-1821), CD30 (sense, 5'-CTG TGT CCC CTA CCC AAT CT-3', region 1121-1140; antisense, 5'-CTT TTG TCC TTC ATG AGA AAG-3', region 841-825), CD30L (sense, 5'-CCT TTT TCC AGG GCC GAA GAA AAA CTG-3', region 1121-1140; antisense, 5'-CTT TTG TCC TCC CCC CTTCCA-3', region 1980-1960), and β-actin (Clontech Laboratories Inc, Palo Alto, CA; sense, region 578-609; antisense, region 1415-1384). PCR conditions for CD30 and CD30L were 4 minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 1 minute at 68°C (45 seconds at 62°C, 1 minute at 72°C for CD30) and a final extension of 5 minutes at 72°C. In the case of β-actin, amplification was performed for 30 cycles according to the manufacturer’s guidelines. Fifteen microliters of amplified cDNAs were run in 1.5% agarose gels, blotted onto nylon membranes (Boehringer Mannheim) and hybridized with 2 × 10⁶ cpm/mL of 32P-end-labeled oligoprobes, specifically designed to recognize PCR products. The CD30L and CD30 probes spanned nucleotide positions 684-707 and 1216-1241, respectively.

**In vitro cultures and proliferation assay.** Purified eosinophils (2.0 × 10⁶/mL) were cultured for 12, 24, 48, 60, and 72 hours in IMDM supplemented with 5% FCS in the presence of increasing concentrations GM-CSF (2, 10, 50, and 100 ng/mL), IL-3 (2, 5, 10, and 50 ng/mL), IL-5 (1, 2, 5, and 10 ng/mL) or combinations of the same cytokines. All recombinant cytokines were obtained from Genzyme Co (Cambridge, MA)

The CD30⁺ HDLM-2 and Karpas 299 cell lines were used as responsive target cells to investigate the capability of native CD30L on human eosinophils to transduce CD30-mediated growth signals.29 Titrations of purified eosinophils (25-50-100 × 10⁶/well) were incubated in 0.5% porcine cell culture gel (as described)22 and cultured for 72 hours with HDLM-2 or Karpas 299 cells (1 × 10⁶ cells/mL) in 96-well U-bottomed microplates. Similar studies were also performed by using unfixed viable eosinophils. Control experiments were performed in the presence of an excess (10 μg/well) of CD30-Fc fusion protein to specifically block CD30-Fc interaction.
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Fig 1. Expression of CD30L protein and mRNA on purified eosinophils as detected by flow cytometry and RT-PCR. (A) Purified circulating eosinophils from normal donors, patients with hypereosinophilic Syndrome (HES), and from pleural fluid of Hodgkin's disease (HD) patients were stained with the anti-CD30L MoAb M80 (left panels, thick lines) or biotinylated CD30-Fc fusion protein (right panels, thick lines). Thin lines indicate background fluorescence, as determined by staining with isotype-matched control IgGs (left panels) or biotinylated control IgG1 (right panels). (B) Detection of CD30L, CD30 and β-actin mRNA by RT-PCR in purified eosinophils from peripheral blood of a normal donor (N), pleural effusion of a HD patient (HD), and PB from an HES patient (HES). Karpas 299 (CD30+/CD30L-) and DG-75 (CD30-/CD30L+) human lymphoma cell lines were used as positive and negative controls.

RESULTS

Expression of CD30L on purified and tissue eosinophils. Eosinophils from normal donors, HES and HD patients displayed a constitutive expression of CD30L (Fig 1). Purified cells were consistently stained either by the anti-CD30L MoAb M80 and CD30-Fc fusion protein as shown by flow cytometry (Fig 1A). Interestingly, eosinophils from PB and/or pleural effusions of patients with active HD or with HES showed a higher constitutive expression of surface CD30L, compared with eosinophils from normal subjects (Fig 1A). In detail, a mean CD30L staining intensity of $2,639 \pm 143$ MESF was recorded with the M80 MoAb on purified eosinophils from 10 normal donors, as opposed to $3,618 \pm 101$ and $3,694 \pm 85$ MESF ($P = .001$) for eosinophils from HD (n = 5) and HES (n = 4) patients, respectively. Conversely, circulating and tissue eosinophils from either normal donors or patients did not express surface CD30 (data not shown).

Immunocytochemistry of cytopsin preparations with MoAb M80 confirmed the expression of CD30L, with a cytoplasmic staining mainly leaning against the nuclear membrane on the majority of purified eosinophils (not shown).

Expression of CD30L mRNA was also studied by RT-PCR. As shown in Fig 1B, a 689-bp amplified cDNA product specific for CD30L was detectable in purified eosinophils from normal donors, and HD and HES patients. CD30L mRNA was also present in DG-75 cells (positive control) but not in Karpas 299 cells (negative control). Eosinophils from different sources did not display mRNA for CD30, which was conversely found in Karpas 299 cells (positive control).

CD30L expression was also consistently detected on tissue eosinophils from 9 reactive (Fig 2A) and 10 pathologic (Fig 2B) lymph nodes, including AILD (n = 3) and HD (n = 7) cases. Surface expression of CD30L was associated with a disperse granular staining pattern in the cytoplasm of the great majority of eosinophils (Fig 2A-B). In several cells the cytoplasmic staining was more evident along the nuclear membrane. Anti-CD30L MoAb M80 also displayed specific reactivity with nonphagocytosing histiocytes and endothelial cells of postcapillary venules (Fig 2A). Staining of histiocytes was homogeneous and stronger, compared with that of eosinophils and endothelial cells.

Regulation of CD30L expression on cultured eosinophils. To evaluate the effects of cytokines involved in functional
activation of eosinophils for the regulation of CD30L, more than 95% pure eosinophils preparations from normal donors were cultured in the presence of increasing concentrations of IL-5, GM-CSF, and IL-3. A dose-dependent upregulation of surface CD30L was observed upon exposure for 24 hours to GM-CSF and IL-5, which was maximal at concentrations of 100 ng/mL (2.0-fold of net MESF increase) and 10 ng/mL (2.2-fold of net MESF increase), respectively (Fig 3A and B). CD30L constitutive cellular density was enhanced by 1.2- to 1.8-fold at GM-CSF concentrations of 2 ng/mL to 50 ng/mL, and by 1.4- to 1.7-folds at IL-5 concentrations of 1 ng/mL to 5 ng/mL (not shown). IL-5 and GM-CSF displayed a higher capability to enhance the surface density of CD30L on human eosinophils compared with IL-3 (1.3-fold at the peak concentration of 10 ng/mL) (Fig 3B). Higher IL-3 concentrations (up to 50 ng/mL) did not result in further CD30L upregulation (not shown). However, the maximal increase of CD30L specific fluorescence intensity (3- to 4-fold) was obtained by the combination of optimal concentrations of GM-CSF and IL-3 (Fig 3A and B). The addition of IL-5 to the GM-CSF plus IL-3 combination did not further increase CD30L expression (Fig 3B). Cytokine-mediated upregulation of surface CD30L was maximal at 24 hours and sustained for up to 48 to 60 hours (data not shown). These data show that expression of CD30L is highly regulated by physiological cytokines involved in recruitment and functional activation of human eosinophils.

CD30L expressed on eosinophils is functionally active.

To examine if native CD30L expressed at the surface of eosinophils was functionally active on CD30+ target cells, titrations of 0.5% paraformaldehyde-fixed purified human eosinophils (CD30L+) were cocultured with $1 \times 10^5$ HDLM-2 or Karpas 299 cells/mL. Previous studies have shown that these CD30+ cell lines are able to proliferatively respond to membrane-expressed recombinant CD30L by being either growth-stimulated (HDLM-2) or growth-inhibited (Karpas 299). Biologic responses mediated by native CD30L expressed on eosinophils were detected using the [H]-TdR incorporation assay. As shown in Fig 4, CD30L+ eosinophils induced a dose-dependent proliferation of the HD-derived cell line HDLM-2 and inhibited growth of the ALCL cell line Karpas 299. The CD30L-mediated proliferative effects of eosinophils appeared to be specific because both growth stimulatory (HDLM-2) and inhibitory (Karpas 299) effects could be blocked by the addition of an excess (10 µg/mL) of soluble CD30-Fc fusion protein (Fig 4). Similarly, viable
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*Typical of HD.* In the present study we have shown that human circulating eosinophils display CD30L mRNA and surface protein. In addition, immunohistochemical staining with the anti-CD30L MoAb M80 indicated that most of tissue eosinophils in reactive and HD-involved lymph nodes express immunodetectable CD30L in vivo. The constitutive surface density of CD30L on eosinophils from HD and HES patients was significantly higher compared with normal donors, probably reflecting a cytokine-mediated activation of eosinophils in these pathologic conditions. In addition, we have shown here that the concerted action of cytokines leading to eosinophil functional activation, ie, IL-5, IL-3, and GM-CSF may concurrently upregulate CD30L at the surface of cultured normal eosinophils. Previous studies have shown a selective enhancement of IL-5, IL-3, and/or GM-CSF levels in sera and tissues of patients with primary or secondary hypereosinophilia and HD, even though cytokine serum concentrations were overall lower than those eliciting maximal CD30L upregulation on cultured normal eosinophils. However, systemic cytokine levels may not reflect concentrations that are obtained in vivo at sites relevant for biologic activity, and it has been recently suggested that local concentrations of cytokines might be much greater than expected. Therefore, it is conceivable that higher tissue concentrations of IL-5, IL-3, and GM-CSF may be actually achieved within the HD microenvironment, at the sites of contact-dependent interactions of H-RS cells with surrounding reactive cells, including eosinophils.

To evaluate whether native CD30L on human eosinophils was functionally active, the CD30⁺ cell lines HDML-2 (HD) and Karpas 299 (ALCL) were incubated in vitro with paraformaldehyde-fixed or viable purified eosinophils. We were able to show that CD30 engagement by CD30L-expressing eosinophils, resulted in the dose-dependent proliferation of HDLM-2 cells and in the growth inhibition of Karpas 299 cells. Native CD30L on human eosinophils is a functionally active surface structure able to transduce CD30-mediated growth signals.

**DISCUSSION**

Even though the presence of a prominent tissue eosinophilia represents a common histopathologic feature of HD, eosinophils have been mainly regarded as “innocent” bystanders recruited and activated during the cellular reaction typical of HD. In the present study we have shown that human circulating eosinophils display CD30L mRNA and surface protein. In addition, immunohistochemical staining with the anti-CD30L MoAb M80 indicated that most of tissue eosinophils in reactive and HD-involved lymph nodes express immunodetectable CD30L in vivo. The constitutive surface density of CD30L on eosinophils from HD and HES patients was significantly higher compared with normal donors, probably reflecting a cytokine-mediated activation of eosinophils in these pathologic conditions. In addition, we have shown here that the concerted action of cytokines leading to eosinophil functional activation, ie, IL-5, IL-3, and GM-CSF may concurrently upregulate CD30L at the surface of cultured normal eosinophils. Previous studies have shown a selective enhancement of IL-5, IL-3, and/or GM-CSF levels in sera and tissues of patients with primary or secondary hypereosinophilia and HD, even though cytokine serum concentrations were overall lower than those eliciting maximal CD30L upregulation on cultured normal eosinophils. However, systemic cytokine levels may not reflect concentrations that are obtained in vivo at sites relevant for biologic activity, and it has been recently suggested that local concentrations of cytokines might be much greater than expected. Therefore, it is conceivable that higher tissue concentrations of IL-5, IL-3, and GM-CSF may be actually achieved within the HD microenvironment, at the sites of contact-dependent interactions of H-RS cells with surrounding reactive cells, including eosinophils.

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cells. These effects were highly specific being abolished by an excess of soluble CD30 protein and completely overlapping the biologic activity of recombinant human CD30L. 24-26

Our finding that eosinophils represent an other important cellular source of functionally active CD30L for H-RS cells and/or other reactive cells within the HD microenvironment, further raises the issue of the pathobiologic significance of eosinophilic infiltration in HD tissues. Expression of CD30L by reactive cells, such as activated T lymphocytes, 11,18-19,31 and eosinophils, may represent a central regulatory event in HD, due to ability of this ligand to transduce CD30-mediated proliferation and anti-apoptotic signals at the surface of H-RS cells. 23-25,27,31 Whether eosinophil-derived CD30L may actually regulate H-RS cells growth in vivo remains to be established, but our in vitro data point in such a direction. Interestingly, other receptors (ie, CD95/Fas) and ligands (ie, CD40L) of the TNF superfamily, have been recently shown to be expressed on circulating normal eosinophils. 32-40 However, even though H-RS cells express CD40 at a high surface density, 32,41 tissue section data for CD40L expression on eosinophils in HD-involved lymph nodes is lacking, and recombinant CD40L does not stimulate H-TdR incorporation of HD cell lines in vitro. 18,31 In addition to H-RS cells, reactive T lymphocytes may represent another putative CD30+ cellular target for eosinophil-expressed CD30L. Expression of CD30 has been recently related to a subset of activated CD4+/CD45RO+ memory T cells and Th2-type T-cell clones, capable of secreting high amounts of interferon-γ (IFN-γ), IL-2, and IL-5. 42-45 It is also clearly established that CD4+ T cells are an important source of most soluble mediators (ie, IL-5, IL-3, and GM-CSF) enhancing eosinophilic maturation, survival, and functional activation. 8,10,14 Intriguingly, CD4+/CD45R0+ T cells represent the main reactive cellular compartment surrounding and functionally interacting with H-RS cells, 46 and in vivo expression of CD30 on this T-cell subset was recently detected by our group in HD-involved tissues. 34 Therefore, it appears likely that eosinophils recruited and activated by H-RS- and T-cell-derived cytokines (IL-5, IL-3, GM-CSF), may in turn interact with these cells, and contribute with both membrane-bound ligands (CD30L, CD40L) and soluble cytokines (TGF-β, GM-CSF, IL-1, IL-3, IL-5, IL-6, and IL-8) to the deregulated cellular reaction underlying tumor cell growth and survival in HD. 2,11,13,18

The prognostic relevance of eosinophilia in HD appears controversial. 1,5,8,10 In this regard, although PB eosinophilia has been considered as a good prognostic indicator, 47,48 a massive eosinophilic infiltration into HD tissues was found to be associated with a poor clinical outcome. 49 In addition, a more recent study specifically addressing the prognostic significance of tissue eosinophils, has shown, by a multivariate statistical approach, that an heavy tissue eosinophil infiltration represented the most important negative prognostic factor affecting disease-free survival in a series of 140 primary HD patients, treated according to a prospective protocol. 50 Should this latter observation be confirmed by other clinical series, HD again will turn as a tumor with unique biologic properties. Usually, a prominent eosinophilic infiltration within tumor tissues rather predicts a favorable outcome in other malignancies including cervix, lung, gastric, and colorectal carcinomas. 9,10 Whether eosinophil-mediated activation of the CD30 system on either H-RS cells or other reactive cells (ie, CD4+ T cells) may have a role in HD progression remains to be established. Interestingly, however, in nodular lymphocyte predominance HD, where eosinophilia is a very rare occurrence, 5,60 H-RS cells do not usually express CD30 and the disease shows a prolonged and indolent clinical course. 2,13,23,51

Our present data suggest that eosinophils may act as important elements in the pathology of HD by contributing to the deregulated network of CD30/CD30L-mediated interactive signals between H-RS cells and surrounding reactive cells.

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