Expression and Function of CD40 on Hodgkin and Reed-Sternberg Cells and the Possible Relevance for Hodgkin’s Disease

By Hans-Jürgen Gruss, Daniel Hirschstein, Barbara Wright, Dawn Ulrich, Michael A. Caligiuri, Maurice Barcos, Laura Stockbline, Richard J. Armitage, and Steven K. Dower

CD40 was originally described as a B-cell–restricted antigen and was subsequently found to be a member of the tumor necrosis factor (TNF) receptor superfamily. CD40 is also expressed on dendritic cells, thymic epithelium, monocytes, and some carcinoma cell lines, and plays a critical role in cell contact-dependent activation. Primary and cultured Hodgkin and Reed-Sternberg (H-RS) cells, the presumed malignant cells of Hodgkin’s disease (HD), were found to express high levels of cell surface CD40. We found that recombinant CD40 ligand (CD40L) induced interleukin-8 (IL-8) secretion and enhanced IL-6, TNF, and lymphotoxin-α (LT-α/TNF-α) release from cultured H-RS cells. These cytokines play a significant role in the clinical presentation and pathology of HD, a tumor of cytokine-producing cells. CD40L had no mitogenic activity for HD-derived cell lines. In contrast, CD40L enhanced expression of costimulatory molecules intracellular adhesion molecule-1 and B7-1 on cultured H-RS cells, both of which are overexpressed on primary H-RS cells. In addition, CD40L induced a 40% to 60% reduction of the expression of the HD-associated CD30 antigen, another member of the TNF receptor superfamily. Primary and cultured H-RS cells express not only CD30, but also CD40. CD40L has pleiotropic biologic activities on H-RS cells, and the CD40-CD40L interaction might be a critical element in the deregulated cytokine network and cell contact-dependent activation cascade typical for HD.

© 1994 by The American Society of Hematology.

HODGKIN’S DISEASE (HD) is defined as a group of malignant lymphomas with common clinical and pathologic features. The diagnosis of HD is typically based on a disrupted lymph node architecture and the presence of the presumed malignant monoclonal Hodgkin and multinucleated Reed-Sternberg (H-RS) cells embedded in an abundance of reactive cells (eg, lymphocytes, histiocytes, eosinophils, and plasma cells) without malignant transformation. The etiology of HD and the origin of the H-RS cells remains unclear. Primary and cultured H-RS cells express a heterogeneous panel of cytokines and cytokine receptors, which correlate with typical clinical and pathologic presentation of HD cases. An unbalanced production of cytokines in active HD has been associated with constitutional ‘‘B’’ symptoms with fever, night sweats, generalized itching and weight loss (interleukin-1α [IL-1α] tumor necrosis factor [TNF] and lymphotoxin-α [LT-α]), thrombocytosis (leukemia-inhibitory factor [LIF], IL-6, and IL-11), increased alkaline phosphatase serum levels (macrophage colony-stimulating factor [M-CSF]), elevation of serum acute-phase reactants (IL-6, LIF, IL-1α, and IL-11), blood and tissue eosinophilia (IL-5, granulocyte-macrophage CSF [GM-CSF], and IL-3), sclerosis of HD-involved tissues (transforming growth factor-β [TGF-β and LIF]), decreased immune responses (IL-10 and TGF-β), and T-cell rosetting around H-RS cells (IL-9, CD30L, IL-6, TNF, LT-α, and the cytokine regulated adhesion molecule intracellular adhesion molecule-1 [ICAM-1]).

The CD40-CD40 ligand (CD40L) interaction has been identified as a critical signal for cell contact-dependent T-cell activation of B cells. The 50-kd CD40 molecule is expressed on B cells, dendritic cells, monocytes, some carcinoma cell lines, and thymic epithelium and is a member of the TNF receptor superfamily. This receptor family includes the low-affinity nerve growth factor (NGF) receptor, two distinct receptors for TNF, soluble viral TNF receptors, CD27, CD30, OX40, 4-1BB, and the Fas antigen. Ligands have been identified for the TNF receptors and NGF receptor, and more recently for CD40, CD27, CD30, 4-1BB, and Fas. CD40L expression is restricted primarily to T cells, mainly the CD4+ subpopulation, which accumulate as reactive T cells in HD-involved tissues, surrounding H-RS cells in a rosette fashion. More recently, CD40L expression has been reported on some mast and basophil cell lines. Signaling through CD40 mediates activation and proliferation of B cells and is essential for Ig isotype switching. In addition, CD40L is stimulatory for monocytes and T cells in vitro. Other cell contact-dependent interactions after antigenic stimulation are mediated by lymphocyte function associated antigen-1 (LFA-1)/ICAM-1 (CD54) and B7-1 (BB-1)/CD28-CTLA-4, the expression of which is enhanced after CD40 ligation. H-RS cells express high levels of several cytokine receptors, eg, CD25 (IL-2R p55), CD30, CD71, IL-2R p75, IL-6R, and adhesion molecules such as ICAM-1. We report here the abundant expression of CD40 by primary and cultured H-RS cells. In addition, culture of H-RS cells with soluble trimeric CD40L or surface-expressed human CD40L results in the enhanced secretion of cytokines (eg, TNF, LT-α, IL-6, and IL-8) and upregulation of costimulatory molecules (eg, B7-1) and adhesion molecules (eg, ICAM-1), all known to play a significant role for HD. Thus, CD40 is another cytokine receptor expressed at high levels.
by primary and cultured H-RS cells, and may prove to be a critical element in the cytokine and cell contact-dependent activation network for the pathology of HD, a tumor of cytokine-producing cells.

MATERIALS AND METHODS

Cells and culture conditions. Peripheral blood T (PB T) and tonsil B cells were isolated as described previously.24 Purified T- and B-cell preparation were always greater than 98% CD3+ or CD20+, respectively, as determined by flow cytometry. The HD-derived permanent cell lines HDLM-1, -2, or -3, L-428, KM-H2 (kindly provided by Dr H.G. Drexler, German Collection of Microorganisms and Cell Culture, Braunschweig, Germany), and L-540 (kindly provided by Dr V. Diehl, University of Cologne, Cologne, Germany) are Epstein-Barr virus negative (EBV+) tumor cells from HD patients and have been characterized extensively.25 These HD-derived cell lines are generally accepted to be representative of the neoplastic component of HD based on many characteristics in common with primary H-RS cells. The CD30+ large-cell anaplastic lymphoma (LCAL) cell lines were described previously.26 Karpas299 cells (kindly provided by Dr H.G. Drexler) and DHL-1 cells (kindly provided by Dr A. Lorenzoza, Hospital of Sick Children, Toronto, Canada) have the same cytochemical, immunologic, morphologic, and chromosomal profile as the original tumor cells of the LCAL patients. Cells were cultured in RPMI1640 (GIBCO, Gaithersburg, MD) supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine (Sigma Chemical Co, St Louis, MO) as suspension cultures at 37°C, in a humidified atmosphere of 7% CO2.

Reagents. Soluble trimeric human CD40L was obtained from COS cells transfected with a construct consisting of the extracellular domain of CD40L fused to a modified leucine zipper motif27 Supernatants were harvested 4 days posttransfection and concentrated 10-fold using Centriprep 10 concentrators (Amicon, Danvers, MA), as described previously.11 Concentrated supernatants were stored at −70°C before use. CV-1/EBNA cells were transfected using the diethyl aminoethyl (DEAE)/Dextran method with either the expression vector alone (CV-1/HAV) or vector containing the human (hu) CD40L cDNA (CV-1/CD40L) and then fixed 2 days posttransfection with 1% paraformaldehyde for 5 minutes at 25°C. Expression levels of CD40L were determined by flow cytometry using soluble CD40Fc-fusion protein as described previously.28

Antibodies and flow cytometry. Expression of surface antigens was examined by indirect immunofluorescence using flow cytometry (FACScan, Becton Dickinson Corp, Sunnyvale, CA) as described previously.8 The following monoclonal antibodies (MoAbs) were used for flow cytometry and immunohistochemistry as indicated. Murine MoAbs specific for B-1 (IgG2a, anti-CD38; L304.4), CD18 (IgG2a, anti-LFA-1, CD11a; L130), CD44 (IgG1, anti-HCAM; L178), CD56 (IgG1, anti-NCAM; MY31), CD58 (IgG1, anti-LFA-1, L306.4), and LECAM-1 (IgG1, SK11) were purchased from Becton Dickinson (San Jose, CA). CD11a (IgG1, anti-LFA-1, CD11a; 25.3.1), CD25 (IgG1, anti-IL-2R α chain; 3B3.1), CD28 (IgG1; CD28.2), CD54 (IgG1, anti-ICAM-1; 8H10), CD57 (IgG1; NC-1), and CD70 (IgG1, anti-CD27L, Ki-24) were obtained from Amgen Inc (Westbrook, ME). Mouse IgG1 or mouse F(ab')2 polyclonal antimouse IgGs conjugated with R-phycocerythrin (PE) were obtained from Tago (Burlingame, CA). The murine anti-CD40 MoAbs M2 and M3, raised against soluble CD40Fc, have been shown to block binding of CD40 ligand to CD40 and, after cross-linking to induce proliferation of tonsil B cells.21 The clones producing monomolecular mouse antihuman nonblocking CD30 MoAbs (M67 and M3) or mouse antihuman nonneutralizing CD40L MoAbs (M79) were produced against soluble CD30Fc and cell surface expressed human CD40L, respectively.20

Proliferation assay. HD-derived cell lines were cultured in the presence of soluble and immobilized anti-CD40 MoAbs (M2 and M3) or isotype-matched control MoAb (IgG1), or with a titration of fixed CV-1/EBNA cells transfected with vector alone or huCD40L. Cells were also cultured with a 1:10 (vol/vol) soluble trimeric huCD40L or COS cell control supernatants. Cultures were pulsed after different culture times with 1 µCi/well 3H-thymidine (specific activity, 25 Ci mmol; Amersham, Arlington Heights, IL) for the final 12 hours of culture. Incorporated cpm were determined by tritium sensitive avalanche gas ionization detection on a Maxic 96 Beta Counter (Packard, Meriden, CT).

RNA extraction and Northern blot analysis. RNA was extracted from the HD-derived cell line and stimulated tonsil B and PBL cells using the acid-guanidinium thiocyanate-phenol-chloroform method29 and fractionated by electrophoresis through a 1% formaldehyde agarose gel, transferred to synthetic membranes (Schleicher and Schuell, Keene, NH) using capillary blotting, prehybridized and hybridized with a minimum of 106 cpm/mL of specific antisense riboprobes of the huCD40, CD40L, or GAPDH cDNA labeled with 32P-CTP (>6,000 Ci/mmol; Amersham) as described previously.12 The blots were washed at 65°C in 0.1% sodium dodecyl sulfate (SDS)/0.1× SSC and were autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

Immunohistochemistry. Lymph node sections from 13 HD patients with nodular sclerosis (NS) and mixed cellularity (MC) histologic subtype and 3 CD30+ LCAL patients were kindly provided by the Department of Pathology of the Roswell Park Cancer Institute (Buffalo, NY). Most tissue samples were snap-frozen in liquid nitrogen-cooled isopentane and stored at −70°C until used for immunohistochemistry. Acetone-fixed 4-µm cryostat sections were used. Some tissue samples were paraffin-embedded and formalin-fixed using a automatic tissue processor. Tissue sections (5 µm) were dewaxed using standard Xylo and alcohol procedures. A three-step avidin-biotin complex (ABC) technique was used for immunodetection.23 The ABC-complex was peroxidase-conjugated (Vector Laboratories Inc, Burlingame, CA). Equal volumes of 0.1% 3,3′-diaminobenzidine (DAB) tetrahydrochloride and 0.02% hydrogen peroxide were used as peroxidase substrates (Vector). Controls consisted of replacement of the primary antibody by an irrelevant isotype-matched MoAb or use of chromogen alone.

Cytokine assays. IL-6 and TNF (TNF-α) secretion by HD-derived cell lines was measured by enzyme-linked immunosorbent assay (ELISA) as described previously.24 LT-α (TNF-β) and IL-8 concentrations were detected using commercially available ELISA kits (R&D Systems), following procedures recommended by the manufacturer.

RESULTS AND DISCUSSION

Expression of CD40 and CD40L by cultured and primary H-RS cells. Six HD-derived cell lines were examined for cell surface and mRNA expression of CD40 and CD40L using flow cytometry and Northern blot analysis. As shown in Table 1 and Fig 1, all 6 HD-derived cell lines expressed CD40 at the protein and mRNA level. Five of six HD-derived lines expressed abundant amounts of CD40 on the surface, with 73% to 100% cells positive and a specific mean fluorescence intensity (sMFI) of 58 to 95 channels assessed by indirect immunofluorescent staining.
with the anti-CD40 M2 MoAb (Fig 1). The CD40 expression on these five HD-derived cell lines was as strong as staining for the CD30 antigen, a marker frequently expressed on H-RS cells (Fig 1). In contrast, L-540 cells were only 32.2% positive with an sMFI of 49 channels for CD40 expression, but 100% positive for CD40L expression at an sMFI of 750 channels (Fig 1). In hyperreactive tonsil, CD40 protein expression was found mainly in typical B-cell areas, but was weaker than that seen on most H-RS cells (Fig 2). The strong expression of CD40 on primary H-RS cells, the presumed neoplastic cells of HD and anaplastic lymphoma cells, resembles that of CD30, another member of the TNF receptor superfamily.2,20,27 High-level CD40 expression was found on phenotypically “T-cell-like” and “B-cell-like” primary and cultured H-RS cells (Tables 1 and 2). These observations are compatible with the recent finding that PBT cells express CD40 on their surface and use CD40L as a costimulatory signal.13 Studies of CD40 expression in other T-cell non-Hodgkin’s lymphomas (NHLs) are needed to define the significance of CD40 expression for a range of lymphomas. It is of interest that very recently in another study, 26 of 37 HD cases showed strong CD40 expression on the primary H-RS cells, but only 3 of 23 NHL cases (1 T-cell and 2 B-cell NHLs) were weakly CD40+.28 As shown in Fig 2 and Table 2, primary H-RS cells did not express CD40L, but scattered lymphoid cells in disease active areas of HD were CD40L+, as shown by immunohistochemistry, using the M79 anti-CD40L MoAb or soluble CD40Fc protein. The scattered CD40L+ lymphoid cells in the HD-involved areas were mainly in the parafollicular areas and in close proximity to the CD40+ H-RS cells (Fig 2). The number of CD40L+ lymphoid cells was increased in the HD cases compared with the minimal CD40L expression observed in normal lymphoid tissues (eg, lymph nodes and tonsils). Only some T cells surrounding H-RS cells were positive for CD40L expression. The expression of CD40L as a surface molecule on T cells is time-dependent and strictly regulated.8,9 It remains to be seen which regulation pathways are altered in HD and could be responsible for increased CD40L expression by T cells surrounding H-RS cells. CD40L expression

Similarly, primary H-RS cells in all 13 HD cases studied with NS or MC histological subtype, as well as the malignant cells in three cases of LCAL, expressed high amounts of CD40 on their surface (shown in Fig 2 and summarized in Table 2). In addition, some cytoplasmic reactivity with the anti-CD40 MoAb M2 was also observed in most malignant cells (Fig 2). In hyperreactive tonsil, CD40 protein expression was found mainly in typical B-cell areas, but was weaker than that seen on most H-RS cells (Fig 2). The strong expression of CD40 on primary H-RS cells, the presumed neoplastic cells of HD and anaplastic lymphoma cells, resembles that of CD30, another member of the TNF receptor superfamily.2,20,27 High-level CD40 expression was found on phenotypically “T-cell-like” and “B-cell-like” primary and cultured H-RS cells (Tables 1 and 2). These observations are compatible with the recent finding that PBT cells express CD40 on their surface and use CD40L as a costimulatory signal.13 Studies of CD40 expression in other T-cell non-Hodgkin’s lymphomas (NHLs) are needed to define the significance of CD40 expression for a range of lymphomas. It is of interest that very recently in another study, 26 of 37 HD cases showed strong CD40 expression on the primary H-RS cells, but only 3 of 23 NHL cases (1 T-cell and 2 B-cell NHLs) were weakly CD40+.28 As shown in Fig 2 and Table 2, primary H-RS cells did not express CD40L, but scattered lymphoid cells in disease active areas of HD were CD40L+, as shown by immunohistochemistry, using the M79 anti-CD40L MoAb or soluble CD40Fc protein. The scattered CD40L+ lymphoid cells in the HD-involved areas were mainly in the parafollicular areas and in close proximity to the CD40+ H-RS cells (Fig 2). The number of CD40L+ lymphoid cells was increased in the HD cases compared with the minimal CD40L expression observed in normal lymphoid tissues (eg, lymph nodes and tonsils). Only some T cells surrounding H-RS cells were positive for CD40L expression. The expression of CD40L as a surface molecule on T cells is time-dependent and strictly regulated.8,9 It remains to be seen which regulation pathways are altered in HD and could be responsible for increased CD40L expression by T cells surrounding H-RS cells. CD40L expression

Table 1. Constitutive CD40 and CD40L mRNA Expression of HD-Derived Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Immunophenotype*</th>
<th>CD40</th>
<th>CD40L</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-derived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDLM-1</td>
<td>&quot;T-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HDLM-2</td>
<td>&quot;T-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HDLM-3</td>
<td>&quot;T-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-540</td>
<td>&quot;T-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KM-H2</td>
<td>&quot;B-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-428</td>
<td>&quot;B-like&quot;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stimulated B cells</td>
<td>B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Stimulated T cells</td>
<td>T</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Phenotype and genotype association with T-cell (T) or B-cell (B) lineage. HD-derived cell lines present with some nonfunctional T-cell ("T-like") or B-cell ("B-like") lineage markers.

† CD40 and CD40L mRNA expression was analyzed by Northern blot analysis using radiolabeled antisense riboprobes corresponding to the coding region of the hu CD40 or hu CD40L cDNA, respectively. Similar total RNA loading for all samples was shown with control hybridization. RNA expression: +, positive; -, negative.

‡ PBTs were activated with 50 ng/mL PMA and 500 ng/mL ionomycin overnight. Tonsilar B cells were stimulated with 1% Staphylococcus aureus Cowan for 24 hours.

Fig 1. Flow cytometric analysis of surface CD40 (●), CD30 (○), CD25 (■), and B7-1 (○○) expression on cultured H-RS cells. Cells were stained with MoAbs M2 anti-CD40, M67 anti-CD30, 33B3.1 anti-CD25, and BB1 anti-B7-1, as indicated, followed by PE-labeled goat-antimouse antibodies for indirect flow cytometry. Control samples (---) were stained with isotype-matched control MoAbs to determine background fluorescence levels. Analysis was performed on a FACScan with a minimum of 5,000 cells for each sample. Results are presented in histograms; fluorescence intensity is shown in log10 intervals and is representative of four experiments.

HDLM-2
L-540
KM-H2
L-428

Log Fluorescence Intensity

Relative Cell Number

0 1 2 3 4
Fig 2. Expression of CD40, CD40L, and B7-1 in HD and LCAL. Primary tissue sections were stained with M2 anti-CD40 MoAb, M79 anti-CD40L MoAb, and BB-1 anti-B7-1 MoAb using an ABC-peroxidase technique. H-RS cells express CD40 and B7-1 antigen, but not CD40L (original magnification x 100 or x 600). Scattered lymphoblastoid cells close to H-RS cells in active HD-involved areas were CD40L+. In LCAL cases, CD40L expression by lymphoid bystander cells was seen at a much lower frequency compared with that of HD-involved areas.

By lymphoid cells in LCAL cases was observed at a much lower frequency than in the HD cases (Fig 2). Cultured and primary H-RS cells from HD patients express high levels of the CD40 antigen, but not the counterstructure CD40L, making an autocrine growth loop involving CD40 unlikely.

To investigate a possible paracrine growth signal mediated through CD40L, cultured H-RS cells were treated for different culture times with soluble or immobilized anti-CD40 M2 and M3 MoAbs, surface-expressed hu CD40L (CV-1/huCD40L), soluble trimeric CD40L, or appropriate controls (isotype-matched control MoAb, CV-1 cells transfected with the expression vector only, and COS control supernatants). Cultured H-RS cells did not respond with increased \(^{3}H\)-thymidine uptake with either CD40 MoAbs or CD40L, regardless of the culture conditions, over a 96-hour culture period (data not shown). The few existing HD-derived cell lines represent an in vitro model of the presumed malignant H-RS cells of HD, but CD40 cross-linking had no mitogenic effect. It cannot be excluded that the failure of the CD40\(^+\) H-RS cells to proliferate when cultured with CD40L results from an in vitro loss of response to CD4OL or the need for additional costimulatory components, such as other cytokines or cellular antigens. Further studies in a SCID human lymphoma mouse model will address the CD40L effect on proliferation of H-RS cells in vivo.

CD40L stimulates cytokine secretion from cultured H-RS cells.
CD40 LIGAND EFFECTS FOR HODGKIN CELLS

CD40L induction of IL-8 secretion was rapid, with maximal enhancement after stimulation with soluble trimeric CD40L for 48 hours. The IL-6 secretion, a threefold to sevenfold increase of TNF, and LT-α after 48 hours for TNF with a sevenfold elevation. CD40L was observed after 72 hours for IL-6 with an eightfold increase, after 48 hours for TNF with a sixfold enhancement, and after 72 hours for LT-α with a sevenfold elevation.

Table 2. Summary of Expression of CD40, CD40L, and B7-1 on Primary H-RS Cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Dx</th>
<th>CD40</th>
<th>CD40L</th>
<th>B7-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28/M</td>
<td>HDNS</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>32/M</td>
<td>HDNS</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>21/F</td>
<td>HDNS</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>66/F</td>
<td>HDNS</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>36/M</td>
<td>HDNS</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>19/F</td>
<td>HDNS</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>34/M</td>
<td>HDNS</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>31/M</td>
<td>HDNS</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>21/F</td>
<td>HDNS</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>29/F</td>
<td>HDMC</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>30/F</td>
<td>HDMC</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>31/M</td>
<td>HDMC</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>23/F</td>
<td>HDMC</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>29/F</td>
<td>LCAL</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>33/M</td>
<td>LCAL</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>38/M</td>
<td>LCAL</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: Dx, pathologic diagnosis of tumor tissue; HDNS, HD, nodular sclerosing form; HDMC, HD, mixed cellularity form.

* Tissue section of HD and LCAL patients were stained with M67 anti-CD30 MoAb, M2 anti-CD40 MoAb, M79 anti-CD40L MoAb or CD40Fc, and BB-1 anti-B7-1 MoAb using immunohistochemistry. Staining intensity of H-RS cells was semiquantitatively scaled: -, no staining; +, weak; ++, moderate; ++++, strong staining.

Primary and cultured H-RS cells produce a variety of cytokines (eg, IL-1, IL-5, IL-6, IL-8, IL-9, TNF, LT-α, CD27L/CD70, M-CSF, GM-CSF, TGF-β, and LIF), the known biologic activities of which correlate with typical clinical and pathologic features of HD. It was of interest to determine whether soluble trimeric CD40L or CV-I/EBNA cells transfected with the full-length hu CD40L cDNA could modulate the secretion of cytokines (eg, IL-6, IL-8, TNF, and LT-α) frequently found in HD cases.

Soluble trimeric CD40L (Table 3) induced all four H-RS cell lines to secrete detectable amounts of IL-8 and enhanced secretion of IL-6, TNF, and LT-α, whereas control COS supernatant had no effect. In the four HD-derived cell lines examined, CD40L induced a fourfold to fivefold increase of IL-6 secretion, a threefold to sevenfold increase of TNF, and a threelfold to eightfold increase of LT-α after a 48-hour culture period (Table 3). L-540 cells did not secrete TNF constitutively, or after CD40L stimulation (Table 3). IL-8 was not detectable in the supernatant of HD-derived cell lines cultured with control COS supernatant (Table 3). The addition of soluble trimeric CD40L for 48 hours induced a 15- to 20-fold induction of IL-8 secretion over the threshold detection level (10 pg/mL) of the IL-8 cytokine assay, with the range of IL-8 levels being between 150 and 200 pg/mL (Table 3). As shown in Fig 3 for the HDLM-2 cell line, maximal enhancement after stimulation with soluble trimeric CD40L was observed after 72 hours for IL-6 with an eightfold increase, after 48 hours for TNF with a sixfold enhancement, and after 72 hours for LT-α with a sevenfold elevation.

CD40L induction of IL-8 secretion was rapid, with maximal...
levels observed after 18 to 24 hours and levels remained elevated for up to 120 hours (Fig 3). In contrast, as shown in Fig 3 for HDLM-2 cells, CD40L did not enhance membrane-bound pro-TNF expression. Similar data were obtained for the other three HD-derived cell lines, i.e., L-428, KM-H2, and L-540 (data not shown). To confirm these findings, the HD-derived cell lines were cultured with CV-1/EBNA cells expressing membrane-associated recombinant hu CD40L. As shown in Fig 3 for HDLM-2 cells, both soluble trimeric CD40L and membrane-bound CD40L were capable of inducing or enhancing cytokine production of H-RS cells in a similar fashion.

The specificity of the CD40L effect on cytokine release of H-RS cells was confirmed using soluble CD40Fc and the blocking CD40 MoAb M2. Both antagonists were able to block CD40L-mediated induction (e.g., IL-8) or elevation (e.g., IL-6, TNF, and LT-α) of cytokine secretion by cultured H-RS cells (Table 3, Fig 3, and data not shown). An isotype control MoAb and human IgG1 had no effect on CD40L-mediated cytokine secretion by H-RS cells (data not shown).

Similar data have been published recently showing enhanced cytokine secretion from cultured H-RS cells after 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation. It is of interest that CD40L induces IL-6, IL-8, and TNF production by monocytes and, as shown here with the effects of CD40L on H-RS cells, that this activity cannot be mimicked by CD40 MoAbs in either soluble or immobilized forms. In fact, soluble CD40 MoAb is a potent antagonist of CD40L-induced cytokine secretion from both monocytes and H-RS cells. These findings are in contrast with the costimulatory activity previously described for soluble CD40 MoAb on B cells and immobilized CD40 MoAb on T cells.

IL-6, IL-8, TNF, and LT-α have all been shown to be secreted by primary and cultured H-RS cells and seem to play a significant role in the pathology of HD. A number of clinical and pathologic features of HD are consistent with characteristics of a tumor of cytokine-producing cells. Constitutional “B”-symptoms (e.g., fever, weight loss, and night
sweats), elevation of acute-phase proteins, presence of mild thrombocytosis, or certain histopathologic presentations (eg, sclerosis; polycaryon formation; plasmacytosis; T-cell accumulation, activation and rosetting; and eosinophilia) can be related to abnormal or unbalanced secretion of cytokines. For instance, IL-6 is the major hepatocyte-stimulating factor inducing acute-phase proteins, has thrombopoietin activity, and induces terminal B-cell differentiation. TNF can cause fever, weight loss, and sweats and might be a significant cytokine involved in the development of constitutional “B”-symptoms. TNF also causes elevation of fibrinogen serum levels, also frequently seen in HD. Sclerosis can be associated with TGF-β and LIF production or the presence of eosinophilia with IL-5, GM-CSF, and IL-3 secretion. In summary, CD40L may function as an element of the deregulated cytokine network of HD by causing enhanced cytokine secretion from CD40+ H-RS cells.

**CD40L induction of activation and adhesion molecules.** Optimal T-cell activation requires two distinct signals: first, the interaction between antigen in association with MHC molecules and the CD3-TCR complex; and second, costimulatory signals provided by accessory molecules. Such costimulatory signals can be provided by cytokines (eg, IL-1, IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, TNF, LT-α, CD27L/CD70, CD30L, CD40L, interferon-γ [IFN-γ], 4-1BB, and GM-CSF) or molecules involved in direct cell-cell contact (eg, LFA-1/ICAM-1, LFA-2/LFA-3, CTLA-4, and CD28/B7 family members). For human B cells, enhanced expression of the membrane-associated molecules LFA-1, ICAM-1, and CD30 was seen after CD40 ligation. Interaction of these molecules with their respective counter-structures (ICAM-1, LFA-1, and CD28/CTLA-4, expressed on activated T cells, could contribute to optimal signaling through CD40 and/or regulation of direct cell-cell contact functions at least in part by enhancing or stabilizing adhesion between different cell types. To determine a possible involvement of the CD40-CD40L interaction in the regulation of accessory molecules involved in T cell/H-RS cell interaction, cultured H-RS cells were examined for cell surface expression of adhesion and activation molecules by flow cytometry. Cultured H-RS cells did not express CD11a/CD18 (with the exception of low-level expression on HDLM-2 cells), CD56, or LECAM-1 molecules constitutively (data not shown). The cultured H-RS cells expressed constitutively high levels of CD25 (IL-2R), CD30, CD44, and CD54 (ICAM-1) on their surface and were positive for CD57, CD58, CD70 (CD27L), and B7-1 (Fig 1 and data not shown). The addition of CD40L for 12 to 72 hours did not alter the expression of CD25, CD44, CD54, and CD70 on the surface of the cultured H-RS cells (data not shown). In contrast, CD40L induced a twofold enhancement of ICAM-1 expression and increased twofold to threefold the level of B7-1 expression on all cell lines examined (Table 4). Interestingly, CD30, which is expressed at high levels on H-RS cells, was reduced by 40% to 50% after CD40L stimulation for 48 hours (Table 4). The CD40L effects on the accessory molecules B7-1, ICAM-1, and CD30 were seen for both the “B-cell-like” (eg, KM-H2 and L-428) and “T-cell-like” (eg, HDLM-2 and L-540) forms of cultured H-RS cells (Table 4). As shown in Fig 4, HDLM-2 and L-428 cells stimulated with CD40L showed a maximal enhancement of ICAM-1 after 24 hours and B7-1 after 72 hours, whereas the lowest CD30 surface levels were observed after 72 hours, with 50% to 60% reduction. Similar results were obtained for KM-H2 and L-540 H-RS cell lines (data not shown). The CD40L-induced effects on surface expression of the adhesion and activation molecules ICAM-1, B7-1, and CD30 could be blocked by the addition of CD40Fc or soluble M2 anti-CD40 MoAb (Fig 4, Table 4, and data not shown). The CD30 antigen has been characterized as a marker for H-RS cells and is expressed at high levels on the malignant cells from most HD and some LCAI cases. Further study is required to understand the molecular and biologic significance of CD40L-induced downregulation of

**Table 4. Alteration of Surface Expression of Activation and Adhesion Antigens on Cultured H-RS Cells After CD40L Stimulation**

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Surface Antigen</th>
<th>Medium (48 h)</th>
<th>Control (48 h)</th>
<th>sCD40L (24 h)</th>
<th>sCD40L + sM2 (24 h)</th>
<th>sCD40L (48 h)</th>
<th>sCD40L + sM2 (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDLM-2</td>
<td>CD30</td>
<td>402</td>
<td>415</td>
<td>283</td>
<td>392</td>
<td>250</td>
<td>397</td>
</tr>
<tr>
<td>B7-1</td>
<td>24</td>
<td>29</td>
<td>62</td>
<td>77</td>
<td>31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD64</td>
<td>310</td>
<td>305</td>
<td>560</td>
<td>323</td>
<td>383</td>
<td>309</td>
<td></td>
</tr>
<tr>
<td>KM-H2</td>
<td>CD30</td>
<td>173</td>
<td>161</td>
<td>107</td>
<td>163</td>
<td>96</td>
<td>159</td>
</tr>
<tr>
<td>B7-1</td>
<td>14</td>
<td>15</td>
<td>39</td>
<td>53</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD54</td>
<td>461</td>
<td>449</td>
<td>931</td>
<td>483</td>
<td>804</td>
<td>459</td>
<td></td>
</tr>
<tr>
<td>L-428</td>
<td>CD30</td>
<td>188</td>
<td>197</td>
<td>133</td>
<td>181</td>
<td>92</td>
<td>202</td>
</tr>
<tr>
<td>B7-1</td>
<td>17</td>
<td>17</td>
<td>40</td>
<td>46</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD64</td>
<td>120</td>
<td>119</td>
<td>209</td>
<td>124</td>
<td>179</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>L-540</td>
<td>CD30</td>
<td>701</td>
<td>718</td>
<td>547</td>
<td>693</td>
<td>479</td>
<td>687</td>
</tr>
<tr>
<td>B7-1</td>
<td>16</td>
<td>20</td>
<td>47</td>
<td>53</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD64</td>
<td>68</td>
<td>60</td>
<td>123</td>
<td>57</td>
<td>97</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

The sMFI was calculated as mean channel number by subtracting control fluorescence values. Data are presented as mean channel number. *HD-derived cell lines were stimulated with medium, control COS supernatants, or sCD40L for the time periods indicated. In selected experiments, a 50 μg/mL soluble M2 anti-CD40 MoAb (sM2) was added. The surface antigen expression was analyzed at the same time point for all samples by indirect flow cytometry using the M67 anti-CD30, L307.4 anti-B7-1, and 84H10 anti-CD64 MoAbs or isotype-matched control MoAbs.
CD30 expression on H-RS cells, but it seems possible that a complex interactive signal cascade between different TNF ligand and receptor family members may be involved in the modulation of surface antigens on H-RS cells. Studies with the aim to identify deregulated signaling pathways or metabolic alterations causing the overexpression of CD30, CD40, TNFRs, and, possibly, other family members on H-RS cells need to be performed.

To confirm a previous report that primary H-RS cells were strongly positive for B7 and that the B7/C28 interaction could play a critical role in HD, we examined our CD40 HD and LCAL cases for B7-1 surface expression on primary H-RS cells. As shown in Table 2 and Fig 2, all 13 HD cases and 3 LCAL cases investigated had strong B7-1 protein expression on the primary H-RS cells. The CD40L-mediated increase of B7-1 expression seen on cultured H-RS cells may reflect a regulatory pathway involving B7-1 expression on primary H-RS cells.

Previous data have shown that the CD40/CD40L interaction plays a critical role in T-cell help for B cells, and may be important for other antigen-presenting cells such as monocytes/macrophages. The data presented here show that both primary and cultured H-RS cells express high levels of CD40 but not of CD40L. Cultured H-RS cells express components for maximizing cell-cell contact-dependent function with CD4+ T cells (eg, CD40, B7-1, and ICAM-1), and the counterstructures (CD40L, CD28/CTLA-4, and LFA-1) are found at high levels on activated T cells. Now these components have all been reported to be expressed on primary H-RS cells or on hyperreactive CD4+ T cells, which surround H-RS. It seems likely that these molecules, which are involved in cellular adhesion and activation, act in conjunction with cytokines to provide a network of interactive signals involved in the pathology of HD. It remains to be determined whether an imbalance in expression, and hence biologic activity, of one or more elements of this network plays a critical role in the pathology of HD. For example, tissue overexpression of ICAM-1 and elevated soluble ICAM-1 (sICAM-1) serum levels in HD patients have been reported recently. A series of cytokines (eg, IL-1, TNF, and IFN-γ) have been shown to upregulate ICAM-1 surface expression and to induce shedding of membrane-bound ICAM-1. CD40L could, at least in part, be responsible for ICAM-1 tissue overexpression in HD-involved areas.
and could cause elevated sICAM-1 serum levels in HD patients. The profound T cell/H-RS cell interaction is a typical pathologic feature of HD. The molecular interactions and regulation of activation between these two cell types is presently not well understood, mainly because of the lack of purified primary H-RS cells for functional analysis. Further studies, particularly in the HD-huSCID mouse system, will help to forward our understanding of T cell/H-RS cell interactions in vivo.

In summary, activated CD4+ T cells express LFA-1, CD28, CD30, ICAM-1, and cytokines, such as IL-2, IL-4, IL-6, IL-9, TNF, LT-α, LT-β, CD30L, and CD40L. Primary and cultured H-RS cells express high levels of CD30 and CD40, two members of the TNF receptor superfamily. These cells also express significant levels of ICAM-1, a costimulatory and adhesion molecule, and B7-1, one of the ligands for CD28. The CD40/CD40L-mediated interaction between H-RS cells and T cells can result in the enhanced release of cytokines (e.g., IL-6, IL-8, TNF, and LT-α) with heterogenic involvement in different cytokine-mediated pathophysiologic features of HD, such as constitutional "B"-symptoms (TNF, LT-α, IL-1, and IL-6); thrombocytosis (IL-6, LIF, and IL-11); increased alkaline phosphatase serum levels (M-CSF); eosinophilia (IL-5, GM-CSF, and IL-3); sclerosis of HD-involved areas (TGF-β and LIF); decreased immune responses (TGF-β and IL-10); or T-cell accumulation, activation, and rosetting around H-RS cells (IL-9, CD30L, CD40L, IL-6, TNF, and LT-α). CD40L appears to be another component in the cascade of cytokines involved in HD, in which variable cytokine expression correlates with heterogenous clinical and pathologic presentation. Deregulated activation of H-RS cells and surrounding CD4+ T cells might result from aberrant expression of B7-1/CD28-CTLA-4 and ICAM-1/LFA-1 molecules by cell-cell interaction. The interaction between H-RS cells and T cells in HD-involved tissue areas involves a network of interactive signals with membrane-associated and cytokine-mediated events, of which the CD40/CD40L, B7-1/CD28-CTLA-4, and ICAM-1/LFA-1 pairs may play a critical role in the pathology of HD. Furthermore, CD40 expression appears to be an important clinical, biologic, and pathologic marker for HD.

ACKNOWLEDGMENT

We thank Drs Steven Gillis, David Cosman, Melanie Spriggs, and Douglas Williams for reviewing the manuscript.

REFERENCES

6. Stamenkovic I, Clark EA, Seed B: A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 8:1403, 1989
23. Fanslow WC, Clifford KN, Zappone JD, Alderson MR, Armi-


27. Smith CA, Gruss H-J, Davis T, Anderson D, Farrah T, Baker E, Sutherland GR, Brannan CI, Copeland NG, Jenkins NA, Grabstein KH, Gimpel S, Gillis S, Din WS, Goodwin RG, Armitage RJ: CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. Cell 73:1349, 1993


41. Payne SV, Jones DB, Wright HD: Reed Sternberg cells/lymphocyte interaction. Lancer 2:768, 1977

Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease

HJ Gruss, D Hirschstein, B Wright, D Ulrich, MA Caligiuri, M Barcos, L Strockbine, RJ Armitage and SK Dower