Expression of Functional CD40 Antigen on Reed-Sternberg Cells and Hodgkin’s Disease Cell Lines

By Antonino Carbone, Annunziata Gioghini, Valter Gattei, Donatella Aldinucci, Massimo Degan, Paolo De Paoli, Vittorina Zagonel, and Antonio Pinto

CD40 is a member of the nerve growth factor receptor family, showing a significant homology to the Hodgkin’s disease (HD)–associated antigen CD30 and is capable of transducing growth signals in a number of cell types. A series of 312 lymphoma samples, including 139 cases of HD, 32 cases of CD30+ anaplastic large cell (ALC) lymphomas, and a panel of HD- or NHL-derived cell lines, were evaluated for CD40 expression by immunostaining of paraffin embedded sections, cell smears and by flow cytometry. CD40 was strongly expressed with a highly distinct pattern of staining on Reed-Sternberg (RS) cells and variants in 100% (139/139) of HD cases, irrespective of their antigenic phenotype (T, non T–non B) and histologic subtype of HD. Conversely, CD40 was immunodetected on only one third (12/32; 37%) of ALC lymphoma cases and on 105 of 127 B-cell NHLs. The relative cell density of CD40 on HD cell lines (L-428, KM-H2, HDLM-2) as assessed by flow cytometry was significantly higher than on all other lymphoma cells analyzed. Engagement of CD40 by its soluble ligand (CD40L) enhanced both clonogenic capacity and colony cell survival of HD cell lines. Such effect was potentiated by interleukin-9 costimulation in KM-H2 cells. Finally, we have shown that in vitro rosetting of activated CD4+ T cells to HD cells (L-428) is mediated in part by the CD40/CD40L adhesion pathway. Our data indicate that CD40 is a useful antigen for immunodetection and identification of tumor cells in all subtypes of HD, and suggest that it may play a role in the regulation of RS cell expansion and the contact-dependent interactions of these cells with cytokine-producing T lymphocytes.

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Although CD40 has been extensively studied on B-cell populations, certain carcinomas, and more recently on myeloma plasma cells, little is known about its functional significance in other tumor cell types. We were particularly interested in investigating the expression and functional role of CD40 on Reed-Sternberg (RS) cells, the putative neoplastic cells of HD, owing to its significant homology with the HD-associated antigen CD30. This latter antigen is expressed on the surface of RS cells, usually in association with CD15, in up to 80% of HD cases. For this reason, CD30 is commonly regarded as a valuable diagnostic and clinical marker characteristic for HD and related malignancies, including CD30-positive anaplastic large cell (ALC) lymphomas.

In this study, we wished to define the pattern of expression of CD40 on RS cells and to assess its potential utility for the immunodetection of tumor cells in HD. Furthermore, the differential expression of CD40 and CD40L in HD- and non-Hodgkin’s lymphomas (NHL)-derived human cell lines, and the effects of CD40L on the clonogenic growth of cultured RS cells were evaluated. The role of the CD40/CD40L pathway in mediating T-cell adhesion to RS cell lines was also investigated.

MATERIALS AND METHODS

Tissue samples. The study included tissue samples of 139 cases of HD covering all histologic subtypes, and 173 cases of NHL including 32 cases of CD30-positive ALC lymphoma (Table 1). Nonspecific reactive lymphophasoehytatios and lymph node samples with reactive conditions, such as sarcoidosis (3 cases), and toxoplasmosis (2 cases) were also examined. Tissues were fixed in Bouin solution or formalin. When feasible (38 HD cases, 76 NHL cases, and 17 ALC lymphoma cases), a portion of unfixed tissue was snap frozen in liquid nitrogen and stored at +80°C. Pathologic specimens were classified according to the Working Formulation and to the updated Kiel classification for NHL. The morphologic criteria used to identify ALC lymphomas were described by Aagnarsson and Kadin. The Rye modification of the Lukes and Butler classification was used to classify HD.

Immunostaining. Anti-CD40 MoAb 89 (kindly provided by Dr J. Banchereau, Centre de Recherche Schering-Plough, Dardilly, France) kindly provided by Dr J. Banchereau, Centre de Recherche Schering-Plough, Dardilly, France. 89 was used to classify HD.
CD40 IN HODGKIN’S DISEASE

**Table 1. Expression of CD40 Receptor Protein in Hodgkin’s and Non-Hodgkin’s Lymphomas**

<table>
<thead>
<tr>
<th>Histologic Diagnosis</th>
<th>No. of Positive/Tested Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD*</td>
<td>139/139</td>
</tr>
<tr>
<td>Lymphocyte predominance†</td>
<td>14/14</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td>87/87</td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td>32/32</td>
</tr>
<tr>
<td>Lymphocyte depletion</td>
<td>6/6</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphomas</td>
<td></td>
</tr>
<tr>
<td>B-cell type</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic</td>
<td>17/18</td>
</tr>
<tr>
<td>Follicular center cell</td>
<td>21/22</td>
</tr>
<tr>
<td>Diffuse small cleaved cell</td>
<td>9/10</td>
</tr>
<tr>
<td>Diffuse mixed</td>
<td>5/6</td>
</tr>
<tr>
<td>Diffuse large cell</td>
<td>40/46</td>
</tr>
<tr>
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<tr>
<td>Small noncleaved cell</td>
<td>0/5</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>2/2</td>
</tr>
<tr>
<td>Monocytic B-cell</td>
<td>1/1</td>
</tr>
<tr>
<td>Ki-1/CD30+ anaplastic large cell</td>
<td>5/10</td>
</tr>
<tr>
<td>Extramedullary plasmacytoma</td>
<td>1/5</td>
</tr>
<tr>
<td>Composites</td>
<td>1/1</td>
</tr>
<tr>
<td>T-cell type</td>
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<td>Mycosis fungoides</td>
<td>0/5</td>
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<tr>
<td>Peripheral T-cell</td>
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<tr>
<td>Lymphoblastic</td>
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<tr>
<td>Ki-1/CD30+ anaplastic large cell</td>
<td>2/9</td>
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<tr>
<td>Undetermined</td>
<td>5/13</td>
</tr>
<tr>
<td>Ki-1/CD30+ anaplastic large cell</td>
<td>5/13</td>
</tr>
</tbody>
</table>

* The expression of B-cell associated markers on RS cells was found in 28 HD cases (11 lymphocyte predominance, 12 nodular sclerosis, 4 mixed cellularity, 1 lymphocyte depletion), whereas the expression of T-cell–associated markers was found in 6 cases, all of the NS subtype. The RS cells in the remaining 105 HD cases were classified as undetermined (non-B–non-T, 102 cases; T and B, 3 cases) phenotype.

† The lymphocyte predominance subset included 10 cases of the nodular variant and 4 cases of the diffuse variant.

‡ Small lymphocytic and immunoblastic lymphoma (Richter’s syndrome).

§ One case with lymph node involvement by mycosis fungoides.

France) was applied to paraffin-embedded tissues from all lymphoma cases included in the study. Deparaffinized and cryostat sections were used for further immunophenotyping and lineage assignment of lymphoma cases. Source and specificities of antibodies used in this study have been reported in detail previously.24-26 In addition, anti-BLA.36 MoAb28 (Oncogene Science Inc, Uniondale, NY) was tested on a subset of HD cases (n = 25) including all four subtypes. Anti-BLA.36 MoAb recognizes a 36-kD glycoprotein expressed on a subset of normal B cells, on tumor cells from a fraction of B-NHL, Kupffer cells in normal liver, RS cells and on HD and B-cell lines.27 Immunohistochemistry was performed with the alkaline-phosphatase antialkaline-phosphatase (AAPAP) method as described.28

Cytospin smears of HD- and ALC lymphoma–derived cell lines were fixed in acetone chloroform at room temperature for 10 minutes and immunostained with anti-CD40 and anti-CD30 MoAbs by the AAPAP method.29

**Cell lines.** The characteristics of the human HD cell lines KM-H2, HDLM-2, and L-428 and the Ki-1/CD30+ ALC lymphoma cell line Karpas-299 of T-cell phenotype (CD5+, CD7+) were described in detail previously.29-30 HD- and ALC lymphoma–derived cell lines were compared with other tumor cell lines of B-cell phenotype at different stages of maturation: Sc-1, Cl-1, Ri-1 (B-cell NHL),31 NALM-6 (acute pre-B-cell leukemia), MN60 (SmIg+ acute B-cell leukemia), and U-266 (multiple myeloma). All cell lines were obtained through the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), with the exception of Sc-1, Cl-1, and Ri-1 cell lines that were kindly provided by Dr KH Th'ng (Hammersmith Hospital, London, UK) and Jurkat leukemia T cells obtained from Dr G. Manoletti (University of Trieste, Trieste, Italy).

**Flow cytometry.** Indirect immunofluorescence of cell lines was performed by incubating cells with the same saturating concentration of anti-CD40 MoAb 89, as previously described.26 As a second-step reagent, fluorescein isothiocyanate (FITC)–conjugated F(ab')2 fragments of goat-antimouse Ig (H+L) (Technogenetics, Milan, Italy) were used. Nonspecific binding of MoAbs was assessed by labeling cells with isotype-matched control mouse IgGs (Becton Dickinson, Mountain View, CA). 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). Logarithmic values of fluorescence intensity were converted into the number of molecules of equivalent soluble fluorochrome per cell (MESF)32 by comparing the results of anti-CD40 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.32 Fluorescence intensity achieved with an isotype-matched unreactive antibody was subtracted from that obtained with anti-CD40 MoAbs to calculate the net MESF values.33

**Northern blot analysis and reversed transcriptase polymerase chain reaction (RT-PCR).** Cellular RNA was extracted by the guanidium thiocyanate–phenol-chloroform method.34 RNA samples were size fractionated through a 1% agarose gel containing 2.2 mol/L formaldehyde, capillary blotted onto nylon transfer membranes, and hybridized with 1 to 2 × 108 cpm/mL of 32P-labeled probes of human CD40 (0.46 kb NcoI insert in pGEM-T vector) and CD40L (1.1 kb EcoRI insert in Bluescript vector; courtesy of Dr R.J. Armitage, Immunex R & D Co, Seattle, WA). Filters were washed to a final stringency of 0.1 × standard sodium citrate, 0.1% sodium dodecyl sulfate. For RT-PCR, 1 µg of RNA was reverse transcribed by avian myeloblastosis virus (AMV) reverse transcriptase (Promega Co, Madison, WI) in a 20-µL reaction mix containing hexadeoxyribonucleotides random primers (0.4 µg) for 1.5 hours at 42°C. Five microliters of the same cDNA bulks were amplified in a 50-µL volume of final reaction mix by a Perkin Elmer 9600 thermal cycler, with primers pairs for human CD40 (sense, region 149-168; antisense, region 581-562) and CD40L (sense, region 406-423; antisense, region 697-678). Primer sets were obtained from Stratagene (La Jolla, CA) and amplification was performed according to manufacturer’s instructions.

**Colony assay.** A soluble trimeric form of the naturally occurring ligand for CD40 obtained from supernatants of transfected COS-7 cells, and control COS-7 cell supernatants were kindly provided by M.B. Widmer (Immunex Co). Clonogenic growth of L-428 and KM-H2 cells was assayed as previously described.33 Briefly, 1.0 × 103 cells were cultured in 1 mL of Dulbecco’s modified Eagle’s medium containing 15% fetal calf serum and 0.8% methylcellulose along with different titrations (1:12.5, 1:25, 1:50, 1:100, 1:200, 1:400 vol/vol) of soluble oligomeric human CD40L or COS-7 control supernatants. Cells were cultured in 100-µL aliquots (6 to 8 replicates) in 96-well flat-bottomed microplates. After 7 and 14 days of incubation, aggregates with ≥40 cells were scored as colonies. Results were expressed as percent growth relative to control COS-7 supernatants. In other experiments, cells were preincubated for 2 to 4 hours with different concentrations (0.25 to 10.0 µg/mL) of purified anti-
Fig 1. (A) Hodgkin's disease, LP subtype, nodular variant. Within a nodule containing a large number of small lymphocytes immunostaining with anti-CD40 MoAb 89 discloses the presence of a population of large cells (the LP variants of the RS cell). These cells are characterized by strong membrane labeling associated with diffuse cytoplasmic staining. Note positive staining of surrounding small lymphocytes. Bouin-fixed paraffin-embedded tissue section, APAAP immunostaining, hematoxylin counterstain. (Original magnification × 400.) (B) Hodgkin's disease, mixed cellularity subtype. A RS cell of classical type shows a strong staining for anti-CD40 antibody 89. A very intense staining on the cell membrane is associated with a strong dot-like paranuclear positivity. Bouin-fixed paraffin-embedded tissue section, APAAP immunostaining, hematoxylin counterstain. (Original magnification × 630.) (C) Hodgkin's disease, nodular sclerosis subtype. Many variants of the RS cell (the lacunar cells) may be seen in this field; most cells show a distinct pattern of staining for anti-CD40 antibody 89; a strong membrane staining is associated with a dot-like cytoplasmic positivity. Bouin-fixed paraffin-embedded tissue section, APAAP immunostaining, hematoxylin counterstain. (Original magnification × 320.) (D) Anaplastic large cell lymphoma, CD30 positive with non B-non T phenotype. Only a fraction of anaplastic large tumor cells react with the anti-CD40 MoAb 89. Staining pattern is of membranous type. Dot-like paranuclear staining or cytoplasmic reactivity are not appreciated. Bouin-fixed paraffin-embedded tissue section, APAAP immunostaining, hematoxylin counterstain. (Original magnification × 400.)
CD40 MoAb 89, anti-CD30 MoAbs Ki-1 and HRS-4, and anti-CD32 MoAb 2E1 (Immunotech, Marseille, France) and then assayed for clonogenic growth as described above. As a further control, three different irrelevant purified MoAbs of the same isotype were used. The effects of the simultaneous stimulation with soluble CD40L (1:25 vol/vol) and recombinant human interleukin-9 (IL-9; R&D Systems, MN) (5.0 U/mL) on the clonogenic growth of the IL-9–sensitive HD cell line KM-H2,28 were also analyzed.

Adhesion of CD40L expressing T cells to HD cell lines. Peripheral CD4+ T cells from normal donors were purified by Ficoll-Hypaque centrifugation followed by negative selection with anti-CD19 plus anti-CD8 immunomagnetic beads (Dynabeads, Dynal, Norway) as described,29 and activated to express CD40L by exposure to TPA (10 ng/mL) and of ionomycin (1 μg/mL) for 8 hours.25 A subclone of the Jurkat T-cell line, showing a constitutive expression of CD40L was isolated in our laboratory (Gatti et al, unpublished results, November 1993). Expression of CD40L on both Jurkat T cells and activated CD4+ T cells was assessed by staining with the anti-CD40L MoAb M90 (courtesy of Dr R.I. Armitage) and RNA studies. Spontaneous rosetting of the HD cell line L-428 with either the Jurkat T-cell line and activated CD4+ normal peripheral T cells was assayed as described by Sanders et al.30 Briefly, 1.0 × 10^6 L-428 cells and 10 × 10^6 CD4+ activated T cells or Jurkat T cells were incubated for 1 hour at 37°C in the presence and absence of 10 μg/mL of the following purified MoAbs: anti-CD40 (89), anti-LFA-1/CD11a (TS1/22), anti-ICAM-1/CD54 (84H10), anti-CD2 (39C1.5), anti-LFA-3/CD58 (AICD58), and anti-HLA class I (W6/32). Rosettes, defined as three or more activated CD4+ T cells and five or more Jurkat cells adhering to a single L-428 cell,28 were scored in blind by two different observers by counting 300 cells per each experimental condition.

Statistics. Results of fluorescence and clonogenic assays represent the mean ± SEM derived from at least three different experiments. Statistical significance of differences was evaluated by Student’s t-test.

RESULTS

Expression of CD40 in nonneoplastic lymph nodes. In tissue sections of reactive lymph nodes, anti-CD40 MoAbs showed a pan-B reactivity, staining follicular-mantle and germinal-center B-cells, in addition to interdigitating cells. Anti-CD40 MoAbs were not reactive with a population of larger lymphoid cells, with a characteristic perifollicular distribution pattern, which conversely expressed the CD30 antigen. In both toxoplasmosis and sarcoidosis lymphadenitis, “epithelioid” histiocytes were strongly stained.

Expression of CD40 in HD. Expression of CD40 on RS cells from 139 cases of HD, grouped according to the histologic subtype is shown in Table 1. RS cells were strongly stained by anti-CD40 MoAb 89 in all cases of HD examined, irrespective of the histologic subtype (Fig 1, A through C) and of their antigenic phenotype (T, B, non T–non B) (Table 1). In all cases, 90% to 100% of RS cells and variants were labeled. The staining was usually very intense on the cell membranes (Fig 1B); it was associated with a diffuse cytoplasmic staining of variable intensity and with a strong dot-like pattern in the paranuclear area (Fig 1, B and C). With the exception of the lymphocyte predominance (LP) subtype, almost all cases were found to express the CD15 (93%) and the BerH2 (CD30) (99%) antigens. MoAb anti-BLA.36 reacted with RS cells and variants from all HD subtypes in only a fraction (19/25) of the cases studied.

In most cases, where residual normal lymphoid tissue was present, interdigitating reticulum cells as well as B lymphocytes along with macrophages, epithelioid histiocytes, and endothelial cells were stained by the anti-CD40 MoAb 89.

Expression of CD40 in NHLs. NHL cases were classified histopathologically as shown in Table 1. The expression of B-cell–associated markers was found in 137 of 173 evaluated cases, whereas 23 cases were classified as T-cell lymphomas according to their immunophenotypes, and 13 cases were classified as of undetermined (non T–non B) phenotype (Table 1).

Immunoreactivity of anti-CD40 MoAb 89 in the total series of NHL other than ALC lymphoma is listed in Table 1. As expected, anti-CD40 MoAb reacted with 105 of 127 B-cell lymphomas studied. However, the staining pattern was of moderate intensity, with a homogeneous diffuse staining both on the cell membranes and cytoplasms. Dot-like or punctate paranuclear staining was never observed in B-cell NHL. The CD40 antigen was not detected in all the 14 T-cell lymphomas other than ALC lymphoma.

The results of anti-CD40 reactivity with tumor cells in 32 cases of Ki-1/CD30-positive ALC lymphoma are shown in Table 1. In all cases, 100% of neoplastic cells were stained with BerH2/CD30 antibody, whereas anaplastic: large tumor cells reacted with anti-CD40 MoAbs in only about one third (12/32, 37%) of the cases. Interestingly, CD40-expressing ALC lymphoma cells were of either B-cell (5 cases), T-cell (2 cases), or undetermined phenotype (5 cases) (Table 1). In CD40-positive cases, 50% to 100% of tumor cells were labeled, displaying a moderate to strong staining on the cell membranes. However, staining was less intense than that usually observed in the RS cells of HD, and cytoplasmic staining or dot-like paranuclear positivity was not appreciable (Fig 1D).

Expression of CD40 and CD40L in HD- and ALC lymphoma-derived cell lines. On cytoxin preparations, HD cell lines were consistently stained by anti-CD40 MoAbs irrespective of their antigenic profiles, which encompassed B (KM-H2), T (HDLM-2), and non T–non B (L-428) phenotypes.29 Cultured RS cells were characterized by a strong granular cytoplasmic staining with anti-CD40 MoAbs (Fig 2); on a number of cells, this staining was associated with labeling on the cell membranes, leading to an overall picture closely resembling that observed by us on RS cells in HD tissues. CD40 was not detected on ALC lymphoma cell lines Karpas-299, whereas all cell lines were stained by anti-CD30 MoAbs.

Flow cytometry profiles of HD-, ALC lymphoma–derived, and B-cell leukemia/lymphoma cell lines after staining with anti-CD40 MoAb 89 are shown in Fig 3. Specific fluorescence intensity of CD40 staining in HD-derived cell lines (L-428, KM-H2, HDLM-2) was strikingly higher than in all other cell lines tested, which encompassed different stages of B-cell differentiation, from pre-B-cells to plasma cells, and included the ALC lymphoma cell line Karpas-299 (Figs 3 and 4). CD40 staining intensity on HD cell lines ranged from 328,911 to 480,706 MESF, whereas in all other cell types, it ranged from 5,280 to 178,586 MESF (Fig 4). Differences in MESF ± SEM values between each of the three HD
cell lines and any of the other cell types were statistically significant \( (P < .001) \). Differences in CD40 staining were also statistically significant when the mean MESF value of the whole group of HD cell lines \( (421,006 \pm 49,649) \) was compared with that of the whole series of other cell types \( (50,367 \pm 15,258; P < .001) \), to the entire group of NHL cell lines \( (Sc-1, Ci-1, Ri-1, Karpas-299: 85,632 \pm 26,255; P < .001) \), and even to the B-NHL cell lines \( (Sc-1, Ci-1, Ri-1: 118,469 \pm 29,630; P < .001) \).

RNA studies performed with Northern blotting confirmed that all HD-derived cell lines, but not ALC lymphoma cell line Karpas-299, expressed specific transcripts for CD40 (not shown). CD40L mRNA was not detected by Northern blotting in HD cell lines or the ALC lymphoma cell line, but RT-PCR studies evidenced a 292-bp amplified band specific for CD40L in a single HD cell line (HDLM-2) and in the Karpas-299 cell line (data not shown).

Effects of CD40 engagement on colony growth of cultured RS cells. A soluble trimeric form of the naturally occurring ligand for CD40 obtained from transfected COS-7 cells,\(^7\) was tested for its ability to stimulate clonogenic growth of HD cell lines. The values are represented in comparison with control COS-7 supernatants. Exposure of L-428 cells to different concentrations of soluble human CD40L resulted in dose-dependent enhancement of their clonogenic growth with a maximal stimulation (2.5-fold increase; \( P < .01 \)) at a 1:12.5 dilution (Fig 5A). Similar results were obtained after stimulation of L-428 and KM-H2 cells with the anti-CD40 MoAb 89, which produced 3.4- (L-428) and 3.5- (KM-H2) fold increases in the total number of colonies over control cultures at a concentration of 10.0 \( \mu g/mL \) (data not shown). Incubation of L-428 and KM-H2 cells with isotype-matched irrelevant MoAbs, anti-CD30 MoAbs Ki-1 and HRS-4, or anti-CD32 MoAb 2E1 at a concentration of 10.0 \( \mu g/mL \), did not enhance colony formation (not shown). The effects of soluble CD40L and of recombinant IL-9 on the clonogenic growth of the IL-9-sensitive KM-H2 cell line\(^5\) are shown in Fig 5B. CD40L \( (1.25 \text{ vol/vol}) \) was able to produce a 2.2-fold increase in colony formation with respect to cells exposed to control COS-7 supernatants. Such effect was enhanced by recombinant IL-9 \( (5 \text{ U/mL}) \) yielding to 3.9- and 2.3-fold increases in the total number of colonies over cells exposed to control COS-7 supernatants and COS-7 supernatants plus IL-9, respectively \( (P < .001) \). The exposure of L-428 (Fig 6, A and B) and KM-H2 (Fig 6, C and
E) cells to soluble CD40L also resulted in a striking increase in colony size that appeared twofold to threefold larger than in control cultures added with equal concentrations of COS-7 supernatants. The simultaneous exposure of KM-H2 cells to soluble CD40L and recombinant IL-9 (Fig 6F), resulted in the generation of colonies larger than those developed in the cultures added with either IL-9 (Fig 6D) or CD40L (Fig 6E) alone. Finally, colony cells exposed to soluble CD40L displayed an increased in vitro survival (up to 20 days) as compared with control colonies that underwent degeneration starting from the 16th day of culture (data not shown).

Inhibition of T-cell adhesion to HD cells by anti-CD40 MoAbs. To evaluate the involvement of CD40-CD40L pathway in T-cell adhesion to RS cells, rosette formation experiments with the L-428 HD cell line were performed. For such a purpose, purified peripheral CD4+ T cells activated in vitro to express CD40L after an 8-hour incubation with TPA and ionomycin, and a Jurkat T-cell clone expressing CD40L, were used. In both cases, expression of CD40L was monitored by fluorescence staining with the anti-CD40L MoAb M90 (not shown). Up to 35% of L-428 cells were able to bind activated CD4+ T cells (Fig 7). Antibodies to CD40 were able to inhibit rosette formation by more than 60%. Blocking of CD58/CD2 and CD1ldCD54 adhesion pathways by MoAbs resulted in 46% and 89% inhibition of CD4+ T-cell rosetting to L-428, respectively. Simultaneous blocking of all three adhesion pathways (CD40/CD40L, CD1ldCD54, CD58/CD2) by MoAbs, resulted in the complete inhibition of rosette formation (Fig 7). Similarly, anti-CD40 MoAbs were able to produce a 68% inhibition of Jurkat T-cell rosetting to L-428 cells (Fig 7, lower panel). However, simultaneous blocking of CD40/CD40L, CD11a/CD54, and CD58/CD2 adhesion pathways did not result in the complete inhibition of rosette formation of Jurkat T cells with L-428. In all experiments, negative control MoAbs to HLA class I only marginally inhibited rosette formation (Fig 7). Similarly, anti-CD40 MoAbs were able to induce a 55% inhibition of activated CD4+ T-cell rosetting to KM-H2 cells (not shown).

DISCUSSION

Extensive immunophenotypic analysis has shown heterogeneous antigenic profiles on RS cells and there have been no convincing indications of RS cell-specific antigens. Based on these evidences, a combination of several markers, including activation antigens (CD30, CD15, CD25, CD71), HLA-related molecules (CD74, HLA-DR), T- and B-cell-
associated antigens, is currently used for immunodiagnosis of HD in the routine pathology practice. The nodular variant of LP subtype of HD, poses special diagnostic problems because RS cells usually express several B-cell markers, but lack a consistent staining with anti-CD15 and CD30 MoAbs. We have shown that RS cells and their morphologic variants expressed CD40 in 139 of 139 HD cases, irrespective of the histologic subtype and of their antigenic phenotype (B, T, non T–non B). The staining of RS cells with the anti-CD40 MoAb 89 was very intense on the cell membranes being associated with cytoplasmic reactivity and with a strong dot-like pattern in the paranuclear area. Therefore, immunodetection of CD40 on Bouin or formalin-fixed paraffin embedded material appears of high value in the identification of RS cells, including those of the nodular variant of LP subtype. In this latter subtype of HD, the anti-BLA.36 MoAb, recently indicated as a most useful reagent for diagnosing LP HD, reacted with RS cells less consistently than anti-CD40 MoAb 89.

As expected, anti-CD40 MoAb 89 stained most of B-cell NHL, being unreactive in all of the 14 T-cell lymphomas tested. Whereas the distinction between B-NHL and HD may be usually established on immunohistologic findings, it is known that the differentiation of some HD cases of mixed cellularity subtype from peripheral T-cell lymphomas (PTCL) can be difficult in specific cases. PTCL may display RS-like cells and frequently show aberrant deletions of T-cell antigens including CD3, CD2, CD5, CD7, T-cell receptor and even β (TCR,β), while expressing markers such as CD15 and CD30. One may consider the constant expression of CD40 in HD and its absence on T-cell NHL, for discriminating between pleomorphic T-cell lymphomas and a fraction (about 30%) of mixed cellularity HD cases, in which RS cells express T-cell–associated markers such as βF1 (TCR,β), CD2, CD4, and CD3.

The relevance of CD40 expression in making a distinction between CD30+ ALC lymphomas and HD, remains controversial because about one third of ALC lymphoma cases reacted with the anti-CD40 MoAb 89, even though only a fraction of the tumor cells was usually labelled, with a staining intensity lower than in RS cells of HD and without
cytoplasmic or dot-like paranuclear reactivity. However, the absence of CD40 could represent a further tool to rule out HD if the differential diagnosis was HD versus ALC lymphoma.49

By analyzing a limited number of human lymphomas with the MoAb BB-20, O’Grady et al50 have detected CD40 on RS cells in only 26 of 37 HD and in 2 of 12 B-cell NHL cases. These findings are in apparent contrast with our data on HD, and with the present and previous studies indicating that more than 80% of B-cell NHL express CD40.9,10,43-46 Such discrepancies, which appear not to be limited to HD, may be explained in part by an exquisite sensitivity to fixation procedures of the CD40 epitope recognized by the MoAb BB-20, and/or to a low affinity for CD40 of this MoAb. The use of different immunodetection procedures (i.e., flow cytometry v immunohistochemistry and APAAP staining v ABC) should be also taken into account.

The surface density of CD40 on RS cell lines was significantly higher than on all other cell types analyzed in our study. This finding may turn of functional relevance because CD40 engagement may result in active signal transduction,9,12 in the absence of costimulation, provided that the molecule is expressed at a critical threshold level of cell surface density,9,12,14,51 and the lack of growth stimulation by anti-CD40 MoAbs in some B-cell leukemias has been related to a low membrane level of CD40 on tumor cells.5 We have shown here that CD40 engagement by soluble CD40L promotes the clonogenic growth of HD cell lines and enhances their survival in vitro, as indicated by a striking increase in colony size and delayed degeneration of colony cells.

Tumor cell growth can be stimulated by specific growth factors produced by neoplastic cells or by surrounding tissue cells in an autocrine or paracrine fashion. Because only one of the HD cell lines (HDLM-2) displayed CD40L mRNA by RT-PCR, an autocrine loop involving CD40 and CD40L appears unlikely. The typical histologic picture of HD in which RS cells are surrounded by a large number of reactive cells, rather indicates that the strict functional relationship between tumor cells and neighboring tissue components may be more relevant to the pathophysiology of this lymphoma. The membrane-bound form of CD40L14,15,52 is predominantly expressed on activated CD3+/CD4+ cells,12,15,51,52 the same T-cell subset usually surrounding RS cells in the typical microenvironemntal picture of HD.53 These T cells also produce high amounts of IL-9,54 a cytokine specifically involved in the growth of RS cells.76,55 We have shown that spontaneous rosetting of activated CD4+ T cells and of other CD40L+ T cells to HD cell lines, involves in part the CD40/CD40L adhesion pathway. In addition, we have shown a cooperative effect of IL-9 and CD40L in promoting the clonogenic growth of the KM-H2 HD cell line. Therefore, it appears conceivable that, in HD tissues, CD4+/CD40L+ T lymphocytes might bind to RS cells via CD40. Such interaction might result in the transmission of growth and/or antiapoptotic signals to RS cells, and may also favor the action on tumor cells of IL-9 and other T-cell-derived cytokines. The recent report of a high constitutive expression on RS cells of B7/B81 (CD80),65,57 a further adhesion structure for T cells up-regulated on B lymphocytes upon CD40 engagement,58,59 further supports this view.

Our results indicating that CD40 expressed on HD cells is functionally active both as an adhesion molecule and a growth signal transducer suggest that it may play a role in the regulation of RS cells expansion and in the contact-dependent interactions of these cells with cytokine-producing T lymphocytes within the HD microenvironment.

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A Carbone, A Gloghini, V Gattei, D Aldinucci, M Degan, P De Paoli, V Zagonel and A Pinto