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Autocrine Growth Regulation of CD30 Ligand in CD30-Expressing Reed-Sternberg Cells: Distinction Between Hodgkin's Disease and Anaplastic Large Cell Lymphoma

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SUMMARY: Persistent expression of high levels of CD30 in Hodgkin's Reed-Sternberg (H-RS) cells and anaplastic large-cell lymphoma (ALCL) cells, but not in most T- or B-cell lymphomas, suggests the presence of an underlying mechanism leading to the abnormality and possible involvement of CD30 in the growth and survival of these two unique types of tumors. In this study, we examined the effect of CD30 ligand (CD30L) on CD30-positive H-RS and ALCL cells in long-term cultures or in primary cultures. CD30L induced various degrees of proliferation in three long-term cultured H-RS cell lines (L428, HDLM-2, and KM-H2) as well as in primary cultures of H-RS cells obtained directly from patients with Hodgkin's disease. In contrast, significant inhibition was observed in one of the ALCL cell lines (SU-DHL-1), but no growth inhibition or promotion in responding to exogenous CD30L was detected in three others (PB-1, JB-6, and McG-2), nor in primary cultures of ALCL cells. Expression of CD30L was determined by polymerase chain reaction in long-term cultured cells and by an immunohistochemical method in H-RS or ALCL cells de novo in tissue sections. None of the H-RS and ALCL cell lines was positive for CD30L. In tissue sections, we noticed that ALCL cells were generally CD30L-negative. In contrast, the anti-CD30L antibody reacted with a majority of H-RS cells with diffuse cytoplasmic staining. Most H-RS cells were CD30-positive, indicating co-expression of CD30 and CD30L in the majority of lymphoma cells. The persistent high levels of CD30 and CD30L expression in H-RS cells suggest that the autocrine CD30L-CD30 cytokine-receptor loop, in addition to having the paracrine activity previously thought to exist, could play important roles in the proliferation of H-RS cells. In contrast, the CD30L-mediated cytotoxicity may participate in the regression or slow progression of ALCL during the early phase of the disease in selected patients. However, when the disease progresses, the ALCL cells are likely to become resistant to exogenous CD30L. (*Lab Invest 2000, 80:1111–1119*).

In addition to its presence in Hodgkin's Reed-Sternberg (H-RS) cells, CD30 is present in a variety of tumors or activated cells, such as (1) anaplastic large-cell lymphoma (ALCL), (2) rare cases of Lennert's lymphoma, (3) some virally transformed B or T cells or some EBV- or HTLV-1-positive lymphoma cells, (4) a cell line derived from Ph[1]-positive chronic myelogenous leukemia patients in blast crisis (ie, K562 cells), and (5) some B and T cells activated in vitro by mitogens or cytokines (reviewed in Hsu and Hsu, 1994). Comparison of the CD30 cDNA with known sequences indicates that the extracellular domain of CD30 is related to the members of the tumor necrosis factor receptor (TNFR) superfamily, which includes TNFR-1, TNFR-2, low-affinity nerve growth factor receptor (NGFR), CD27, CD40, and CD95 (Fas/APO-1) (Dürkop et al, 1992; Smith et al, 1994).

The fact that CD30 is expressed by both tumor cells and certain activated normal lymphoreticular cells implies that it has a general cell-growth or activation role. However, persistent expression of high levels of CD30 in H-RS cells and ALCL cells, but not in most T- or B-cell lymphomas, further suggests the presence of an underlying mechanism leading to the abnormality and possible involvement of CD30 in the growth and survival of these two unique types of tumors. The latter hypothesis has received strong support from the discovery of a natural ligand for CD30 (CD30L) (Smith et al, 1993).

The reported CD30L is a 239-amino-acid type II membrane protein. It has significant structural similarity to TNF-α, TNF-β, CD40 ligand (CD40L), and Fas ligand (FasL) (Smith et al, 1993). Transcription products of the *CD30L* gene can be detected in B cells, activated T cells, macrophages, granulocytes, eosinophils, and some HTLV-1-positive T-cell lines, but are absent in a number of long-term cultured ALCL and H-RS cells tested (Gattei et al, 1997; Gruss et al, 1994a, 1994b; Gruss and Herrmann, 1996; Nicod and

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Isler, 1997; Pinto et al, 1996; Shanebeck et al, 1995; Wiley et al, 1996; Younes et al, 1996). The recombinant membrane form of CD30L has been shown to exhibit classic pleiotropic cytokine activity. It enhances the proliferation of an H-RS cell line (HDLM-2), yet is cytotoxic to an ALCL cell line, Karpas 299 (Gruss et al, 1994c). The function of CD30L on H-RS cells or ALCL cells in vivo has yet to be determined.

In this study, we examined the effect of CD30L on CD30-positive H-RS and ALCL cells in long-term cultures or, for the first time, in primary cultures. In H-RS cells, increased proliferation was noted upon treatment with exogenous CD30L. For ALCL cells, significant inhibition was observed in a long-term cell line (SU-DHL-1), but no growth inhibition or promotion in responding to exogenous CD30L was detected in three others or in primary cultures. We further examined the expression of CD30L in 21 patients with Hodgkin's disease (HD) and 16 patients with ALCL. We noted a persistent high level of CD30 and CD30L co-expression in H-RS cells, clearly pointing to an important autocrine as well as paracrine (or juxtacrine) loop that may play a fundamental role in the pathogenesis of HD. In contrast to frequent expression of CD30L in H-RS cells, we showed an absence of CD30L in most ALCL cells, indicating a lack of autocrine regulatory activity of CD30L-CD30 in these cells. Escape from paracrine inhibition by CD30L may play an important role in the progression of ALCL.

Results

Production of CD30L-COS-7 and CD30L-293T Cells

We have cloned the full-length coding region of *CD30L* into the pBK-CMV vector in frame with the *lacZ* gene for proper expression in both mammalian cells and prokaryotic cells. In this study, we used the pBKCMV-CD30L plasmid to introduce *CD30L* into COS-7 cells. The polycationic liposome reagent LipofectAMINE ä (Gibco/BRL, New York) was used as transfection reagent, as described by the manufacturer (Gibco). The plasmid for transfection efficiency control was pCMV-β-gal, which expressed β-galactosidase and allowed transfected cells to be stained blue by β-gal. The transfection efficiency was approximately 30–40%.

In addition, we used the full-length coding region of *CD30L* as a template and inserted into the pEGFP-N1 expression vector (CLONTECH, Palo Alto, California). This human CD30L/GFP expression vector was then transfected into 293T cells using the lipofectamine reagent (3 μg of DNA/3 $\times$ 10^5 cells). Forty-eight hours after transfection, the cells were analyzed by fluorescence microscopy and harvested and prepared for studies. The transfection efficiency was approximately 70–80% (Fig. 1, A and B).

Effect of CD30L on H-RS Cells in Short-term Culture and in Primary Culture

CD30L$^+$ COS-7 cells induced various degrees of proliferation in the three H-RS cell lines. Maximal proliferation could be observed by 30%, 82%, and 86% increases in thymidine uptake in L428, HDLM-2, and KM-H2 cells, respectively (Fig. 2, A-C). Vector-transformed COS-7 cells had no effect on the proliferation of cultured H-RS cells. The results were similar to that obtained with CD30L$^+$ 293T cells. Increased proliferation in L428 cells could be demonstrated by a 60% increase in ODs using the MTT/PMS assay. Untransfected 293T cells and vector-transfected 293T cells had no effects on the proliferation of cultured H-RS cells (Fig. 1C). Anti-CD30 (HeFi-1, functions as CD30L) treatment, but not Ber-H2 monoclonal antibody treatment, yielded a similar result. The numbers of cells with BUDR uptake were about the same in both control and CD30L-treated cultures.

Three cases of HD were selected on the basis of the presence of numerous H-RS cells (≅15%) in the tissues. Both un-enriched and H-RS-cell-enriched specimens were available. CD30L induced cell proliferation with an increase in thymidine uptake by 25–45% in un-enriched specimens (containing 80% contaminating lymphocytes and histiocytes) and by 52–100% in enriched specimens (containing less than 5% contaminating cells) (Fig. 2, D-F), indicating that the proliferation was derived primarily from H-RS cells and not from contaminating cells.

Effects of CD30L on ALCL Cells

We showed that the CD30L$^+$ COS-7 cells, CD30L$^+$ 293T cells, or anti-CD30 (HeFi-1) inhibited the proliferation of SU-DHL-1 cells (Fig. 2G), but failed to provoke any significant changes in three other ALCL cell lines (JB-6, MeG-2, PB-1) as well as in two primary

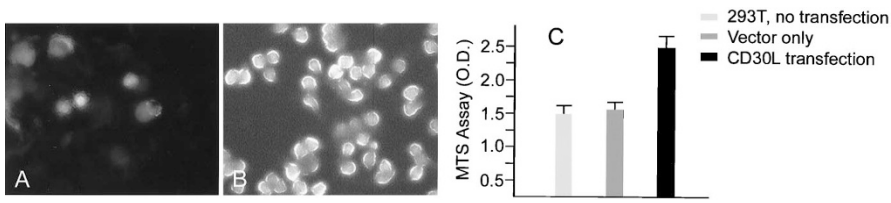

Figure 1.
The human CD30L/GFP expression vector was transfected into 293T cells with efficiency approximately 70–80% (B). Control, untransfected cells were photographed in A. Coculture of H-RS cells (eg, L428 cells) with CD30L-transfected 293T cells resulted in an increased cell proliferation, assayed by a colorimetric non-radioactive MTS/PMS assay. Increase in OD by 60% was noted in cocultures with CD30L$^+$ 293T cells, but not with control 293T cells or vector-transfected 293T cells. Results shown are representative of three independent experiments. Light gray bars: Test cells without co-culture with 293T cells. Gray bars: Test cells treated with vector-transformed 293T cells. Black bars: Test cells cocultured with CD30L$^+$ 293T cells.

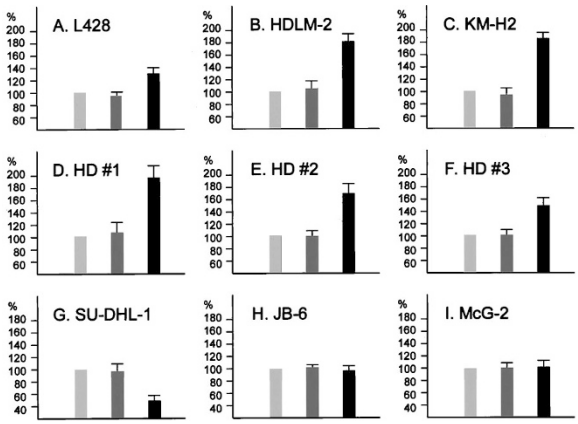

Figure 2.
Biological activity of H-RS cells (A-F) and ALCL cells (G-I) was assessed by co-culture of H-RS or ALCL cells with CD30L$^+$ COS-7 cells. The cells used were (A) L428, (B) HDLM-2, (C) KM-H2, (D-F) H-RS cells from three cases of HD (primary cultures, HD #1, #2, and #3), (G) SU-DHL-1, (H) JB-6, and (I) McG-2. Increased thymidine uptake by 32%, 80%, and 86% was observed in L428, HDLM-2, and KM-H2 cells, respectively. A significant increase in thymidine uptake was also noted in HD #1 (by 100%), HD #2 (by 70%), and HD #3 (by 52%). Decreased thymidine uptake by 54% was observed in SU-DHL-1 cells, but not in other ALCL cells including JB-6, McG-2, as well as PB-1 and two cases of ALCL (not shown). Control experiments were performed by replacement of CD30L$^+$ cells with vector-transformed COS-7 cells. Results shown are representative of three independent experiments. Light gray bars: Test cells without co-culture with COS-7 cells. Gray bars: Test cells treated with vector-transformed COS-7 cells. Black bars: Test cells cocultured with CD30L$^+$ COS-7 cells.

cultures. A 45–60% reduction in thymidine uptake was observed in CD30L- or anti-CD30 (HeFi-1)-treated cultures as compared with the uptake in untreated, or Ber-H2-treated, or vector-transformed cell-treated (control) SU-DHL-1 cultures. In all tests, the numbers of cells and their viabilities remained very similar in both control and CD30L-treated cultures during the first 2 days of culture. Prolonged treatment (up to 2–3 days) of SU-DHL-1 cells with anti-CD30 (HeFi-1) resulted in a 25–30% reduction in the number of cells and an increased number of apoptotic cells.

Expression of CD30L in Cultured Cells

PCR amplification was used for detection of the presence of *CD30L* mRNA in cultured cells (Table 1). A major amplification product of 700 bp was detected in H9, HUT78, and MT-2 (the latter two are HTLV-1-positive) and in PHA-activated human PBL (containing activated T cells). This suggests a possible autocrine role of CD30-CD30L in these cells. The specificity of these PCR products was confirmed by DNA sequence analysis. An additional CD30-/HTLV-1-positive T-cell line (HUT102) was CD30L-negative. None of the H-RS and ALCL cell lines was positive for CD30L. We also found that PHA-activated normal T cells can express CD30 and/or CD30L. We were able to detect CD30 expression in approximately 20–25% of PHA-activated T cells by using immunofluorescence and flow cytometry.

Table 1. Expression of CD30 and CD30L in Cultured Cells

	CD30	CD30L
Nonlymphoblastic T–cell lines		
HUT78	−	+
H9	−	+
HUT102	+	−
MT2	+	+
ALCL cell lines		
SU-DHL-1	+	−
Mcg-2	+	−
PB-1	+	−
JB-6	+	−
H-RS cell lines		
HDLM-1	+	−
L428	+	−
KM-H2	+	−
Normal T cells, PHA–activated		
	+ (15%)	+

Expression of CD30L in Cultured Cells and in H-RS/ALCL Cells in Tissue Sections

In all tissue sections, the anti-CD30L antibody reacted with a portion of the H-RS cells (ranging from 50–70%) with diffuse cytoplasmic staining (Fig. 3, A and B). Membranous staining was not detected, as was also reported by Gruss et al (1996). The monoclonal anti-CD30L antibody was used in frozen sections of five cases of HD, and the resulting staining patterns were similar to those obtained from the polyclonal anti-CD30L in paraffin sections of the respective cases. Diffuse, weak cytoplasmic staining was also seen in the majority of histiocytes. Very weak cytoplasmic staining was detected in scattered lymphocytes. Most H-RS cells were CD30-positive, indicating co-expression of CD30 and CD30L in the majority of H-RS cells.

In 10 of the 16 cases, most ALCL cells were CD30L-negative (Fig. 3, C and D). In four cases, weak and scattered cytoplasmic staining was seen in less than 5% of tumor cells. In the remaining two cases, cytoplasmic staining was observed in approximately 50–75% of lymphoma cells (Fig. 3, E and F). In all tissue sections, the anti-CD30L antibody reacted with a number of histiocytes and granulocytes (ranging from 30–60%) with diffuse cytoplasmic staining (Fig. 3, C to F). Membranous staining was not detected. Very weak cytoplasmic staining was also detected in scattered lymphocytes. The staining obtained with anti-CD30L is specific because it could be inhibited by preabsorption of antibodies with synthetic CD30L polypeptides.

Discussion

The present study showed that the anti-CD30L antibody reacted with the cytoplasm of a subpopulation (up to 70%) of H-RS cells and histiocytes in tissue sections from patients with HD (Fig. 1). The reaction

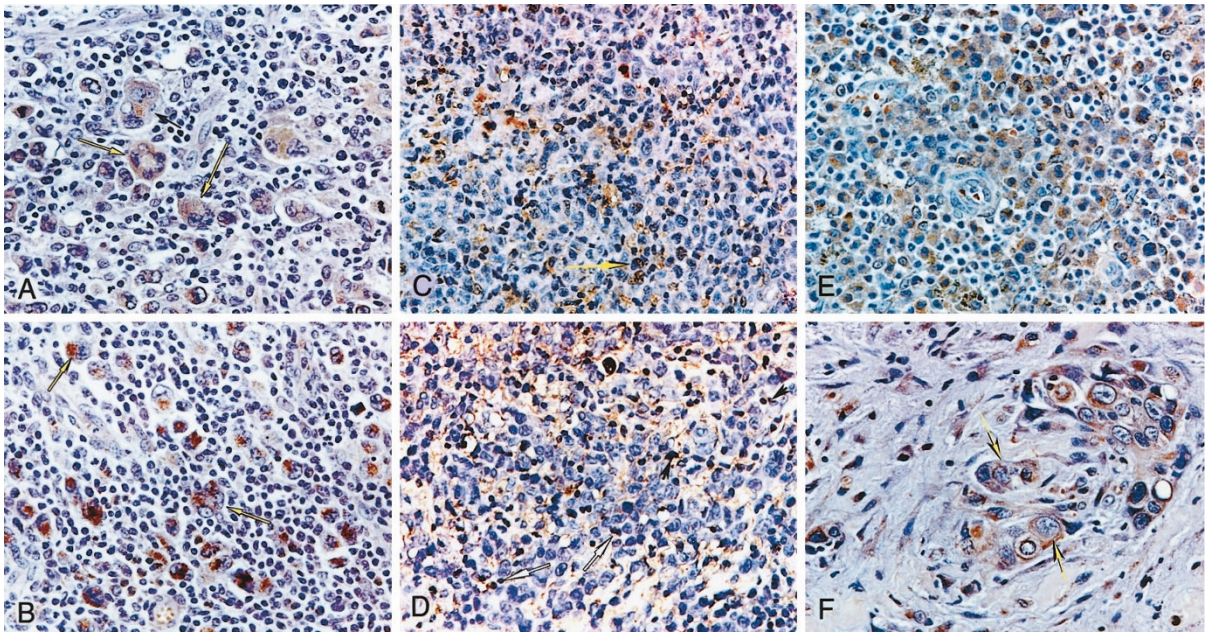

Figure 3.

Expression of CD30L in H-RS cells (A, B). Diffuse cytoplasmic staining was detected in the majority of H-RS cells. Scattered histiocytes and granulocytes were also weakly positive for anti-CD30L staining. The staining in scattered lymphocytes was very weak and was masked after counterstaining with hematoxylin. The great majority of ALCL cells were negative for anti-CD30L staining, as shown in (C) and (D). Arrow in C shows that rare ALCL cells may be CD30L-positive. Most stained cells in C and D were histiocytes or granulocytes. However, in two patients (E and F; both were of null cell phenotype), diffuse cytoplasmic staining was detected in ALCL cells.

with scattered T cells is often weak and is barely detectable after counterstaining. This suggests a rather rich and/or stable source of CD30L from H-RS cells as compared with T cells. The presence of CD30L in CD30-positive H-RS cells in tissues strongly implies an autocrine regulatory role in the survival and/or proliferation of H-RS cells. Although CD30L can be detected in many activated cells, its expression in cultured long-term cell lines is limited to a few Burkitt's lymphoma cell lines (Gruss et al, 1994b). Loss of CD30L in cultured H-RS cells, and other CD30-positive cultured cells as well, may indicate a loss of regulatory dependence in the CD30L-CD30 pathway for these cells.

An auto-regulatory function involving CD30L-CD30 is, however, infrequent in ALCL. Virtually all ALCL cells in long-term culture and the tumor cells in most patients with ALCL did not express CD30L. Expression of CD30L was detected only in two patients among 16 cases examined. The two patients had a rather aggressive clinical course.

The interaction of exogenous CD30L and membrane-bound CD30 has been shown to induce pleiotropic effects on cells, resulting in proliferation, differentiation, or death. Activation-induced death of thymocytes after CD3 cross-linking is impaired in CD30-deficient mice (Amakawa et al, 1996). The CD30L-CD30 interaction, like TNF-R and CD95, is likely to be involved in cell death signaling and participates in negative selection of thymic T lymphocytes. In contrast, both CD30 and CD30L can be expressed by activated T cells, and the CD30-mediated signaling enhances proliferation and cytokine secretion by Th2 cells, and probably by Th1 and Th0 cells (Del Prete et al, 1995; Hamann et al, 1996; Mingari et al, 1996). For B cells, CD30L can stimulate its proliferation, antibody production, and secretion in a cytokine (eg, IL-4 and IL-5)-dependent manner (Shanebeck et al, 1995). Resting and activated B cells express CD30L, and a portion of activated B cells could express CD30 (Trentin et al, 1997; Younes et al, 1996).

Because H-RS cells, and frequently ALCL cells as well, are often in contact with surrounding reactive T lymphocytes and histiocytes, the growth of lymphoma cells may constantly be modulated by the neighboring CD30L-positive cells. Virtually all H-RS cells either obtained from long-term cultures or isolated directly from patient specimens responded to exogenous CD30L to proliferate to various degrees, indicating an important role of the CD30L-CD30 interaction in growth regulation of H-RS cells. Hence, the CD30-CD30L interaction may play a role in initiating and maintaining the neoplastic process by mediating H-RS-T, H-RS-B, and H-RS-eosinophilic contact (Pinto et al, 1996; Trentin et al, 1997).

For ALCL, CD30L may exert a paracrine inhibitory activity on cells of selected ALCL cell lines (ie, SU-DHL-1 and Karpas 299) (Gruss et al, 1994a). The two cell lines (SU-DHL-1 and Karpas 299) (Epstein et al, 1978; Fischer et al, 1988) were derived from patients in advanced stages of ALCL, and yet their growth could still be inhibited by exogenous CD30L. This suggests that a CD30L-mediated inhibition exists not only during the early phase, but also during the late stages of the disease. The clinical course of ALCL is often aggressive (ie, in adults without cutaneous involve-

ment), and the clinical outcome is similar to that of aggressive NHLs (Longo et al, 1995). However, ALCLs occurring in children or confined to the skin seem to run a more favorable course (Kadin et al, 1986; Pileri et al, 1994). These CD30-positive cutaneous T-cell lymphomas (CTCLs) comprise slowly progressive lymphoproliferative disorders [eg, lymphomatosis papulosis (LP)] with various types of evolution, ranging from spontaneous regression to systemic dissemination (Kadin et al, 1986; Pileri et al, 1994; Vecchi et al, 1993). A CD30L expression in CD30-positive neoplastic large cells might have a major role in the mechanism of self-regression of these CD30-positive CTCLs (Mori et al, 1999).

However, the effectiveness of this inhibition in inducing cytotoxicity, cell death, or growth inhibition in many other patients with ALCL may be questioned because an increase in apoptotic lymphoma cells is not a prominent feature in most, if not all, patients, and also because abundant CD30L-positive histiocytes or granulocytes are present in patients without the benefit of tumor regression. Several possibilities may explain how the ALCL cells escape inhibition by exogenous CD30L. First, sCD30 released from the tumor cells (Gause et al, 1991; Josimovic-Alasevic et al, 1989) may compete with membrane-bound CD30 for exogenous CD30L, thus minimizing the effect of exogenous CD30L. Second, the ALCL cells gain growth potential by the expression of growth-promoting molecules (eg, cytokines, cytokine receptors, oncogenes, etc.) and/or by deactivation of tumor suppressor genes. The growth signal delivered could well overcome the inhibitory signal derived from the CD30L-CD30 pathway. Third, the ALCL cells, despite the persistent expression of CD30, may simply become resistant to exogenous CD30L, as we have observed in selected ALCL cell lines.

Whether CD30L expression in ALCL cells as well as the pattern of response by tumor cells to CD30L can be included in the list of prognostic factors for ALCL is a subject of future interest. The prognosis for CD30-positive cutaneous CTCLs may become unfavorable, especially when skin lesions are disseminated, and relapse with systemic involvement may follow an initial spontaneous regression (Kadin et al, 1986; Pileri et al, 1994; Vecchi et al, 1993). Factors such as the presence of t(2;5) or p80 in tumor cells and the clonality of the tumor are being studied as prognostic indicators for the progression of the disease. However, a clonal rearrangement of the T-cell receptor (eg, TcR-γ) does not predict the subsequent development of systemic lymphoma (El-Azhary et al, 1994; Mac Grogan et al, 1996; Sugimoto et al, 1988; Whittaker et al, 1991). Furthermore, the t(2;5) may identify a subset of CTCLs with common cytogenetic features, but the prognostic value of t(2;5) and p80 expression and its associated factors, such as age, immunophenotype, skin involvement, and stage of the disease, may have to be re-evaluated as the methods of detection become more sensitive (Beylot-Barry et al, 1996; Shiota et al, 1994,1995). Future evaluation may also include the expression of CD30L in CTCLs cells (Mori et al, 1999).

The effects of CD30L on short-term cultured H-RS or ALCL cells, other than growth regulation, have not yet been studied systematically. In long-term cultures, the binding of CD30L (and of CD40L as well) to H-RS cells has been shown to affect the expression of cytokines (eg, IL-6, TNF, and lymphotoxin-α) and adhesion/costimulatory molecules (CD54, CD80, and CD86) (Gruss et al, 1994c,1995; Pinto et al, 1996). In a large granular lymphoma cell line, YT, anti-CD30, which behaves like CD30L, has been shown to down-regulate CD28 and CD45 expression and to up-regulate the expression of CD25 (Bowen et al, 1993). The H-RS cells both in tissue and in culture are usually $CD25^+/CD28^-/CD45^-/CD54^+/CD80^+/CD86^+$ (Hsu and Hsu, 1994, 1995). The effects of CD30L on cultured ALCL cells, other than growth inhibition, have yet to be studied. In our study, no significant changes in the phenotypic expression by SU-DHL-1 cells nor by the other three ALCL cell lines have been observed.

In summary, the molecular pathogeneses of HD and ALCL seem to be distinct, despite the numerous cytomorphologic and immunophenotypic parallels that document the close relationship between these diseases. Thus far, our study seems to suggest a distinct CD30L function on H-RS cells (proliferation) and ALCL cells (inhibition). However, it remains a possibility that in a small subset of patients, CD30L exerts an autocrine (self) and/or paracrine regulatory proliferative or survival function rather than an inhibitory function on ALCL cells. It has been speculated that in ALCLs, especially those with t(2;5), the activation of ALK interferes at a pivotal point with the intracellular signal transduction pathway, generating an activated CD30-positive phenotype (Wendtner et al, 1995). In this regard, CD30 expression may be secondary, and paracrine stimulatory effects such as CD30L-CD30 interaction may not be required for cell growth (Wendtner et al, 1995). However, in HD, and perhaps in a rare form of ALCL, autocrine and paracrine mechanisms between CD30L and CD30 seem to be relevant to maintaining tumor cells in an activated state. The phenomenon of paradoxical enhancement of proliferation versus induction of apoptosis is known to be associated with molecules in the TNF/TNF-R family. The exact mechanism is not known (reviewed in Ashkenazi and Dixit, 1998; Lenardo et al, 1999). The signaling of these molecules may be affected by their variable temporal/spatial expression in vivo. The CD30L-dependent proliferation of H-RS cells may be mediated via the Ras/Raf-1/MAPK (ERKII) pathway (Wendtner et al, 1995). Further studies, however, are required for elucidation of the signal transduction pathway for the CD30L-induced cytotoxicity in ALCL cells and of the molecular and cellular events responsible for the regression or slow evolution of a subset of ALCLs.

Materials and Methods

Antibodies, Sources, and Specificities

A polyclonal anti-CD30L antibody was prepared by immunization of goats with synthetic peptide corresponding to amino acids 2–19 mapping to the amino

terminus of CD30L (Santa Cruz Biotechnology, Houston, Texas). A monoclonal anti-CD30L antibody was obtained from Genzyme (Cambridge, Massachusetts). The antibody was prepared by immunization of mice with human CD30L-Fc type II, expressed in CV-1/CHO cells.

Two anti-CD30 antibodies were used for phenotyping and functional assay. The monoclonal antibody HeFi-1 is known to react with the ligand-binding site and can be used as a CD30L-like reagent (Hecht et al, 1985; Tian et al, 1995). The antibody was kindly provided by Dr. R. Fisher (Harvard University, Boston). Another anti-CD30 antibody (Ber-H2), which recognizes a determinant not involved in ligand binding, was used as a control antibody in the functional assay (Younes et al, 1996).

pBK-CMV-CD30L Construct and Preparation of CD30L- and 293T-Expressing COS-7 Cells

COS-7 and 293T cells were used for the eukaryocytic production of CD30L. We used the human full-length CD30L cDNA (pBK-CMV-CD30L plasmid) to transform subconfluent COS-7 cells with the polycationic liposome reagent LipofectAMINE ä (Gibco/BRL). Eight hours later, the cells were trypsinized and reseeded in six-well cluster plates. The cells were grown in culture for 24–72 hours to permit transient expression of the inserted sequence. These cells were fixed with 1% paraformaldehyde for 5 minutes at 25° C and thoroughly washed before use. As control, COS-7 cells transfected with vector alone (pBK-CMV) were used for verification of the specificity of CD30L activity expressed by CD30L-positive COS-7 cells.

In addition, we used the full-length coding region of *CD30L* as a template and inserted into the pEGFP-N1 expression vector (CLONTECH) (Chalfie et al, 1994). This human CD30L/GFP expression vector was then transfected into 293T cells using the lipofectamine reagent according to the manufacturer's instructions (3 μg of DNA/3 $\times$ 10^5 cells). The 293T cells were selected for their high transfection efficacy. The cells were grown in culture for 48 hours to permit transient expression of the inserted sequence. These cells were fixed with 1% paraformaldehyde for 5 minutes at 25° C and thoroughly washed before use. As control, 293T cells or 293T cells transfected with vector alone were used for verification of the specificity of CD30L activity expressed by CD30L-positive 293T cells. For the microscopic analysis of transfection efficacy, the cells were grown on glass coverslips and observed using a Nikon fluorescence microscope with epifluorescence illumination. GFP fluorescence was detected with a Nikon DM510 filter with illumination at 365 nm and long-bandpass emission.

Cell Lines and Cultures

Cells of cell lines including HDLM-1, L428, KM-H2 (these three were HD-derived), SU-DHL-1, PB-1, McG-2, JB6 (these four were ALCL cell lines), and H9, HUT78, HUT102, and MT2 (HTLV-I-positive lines) were cultured in RPMI 1640 medium as previously described (Hsu and Hsu, 1986). The ALCL cell lines (except SU-DHL-1) were kindly provided by Dr. M. Kadin (Harvard University). The nature and phenotypes of these cells have been reported previously (Wasik et al, 1994).

Both COS-7 and 293T cells were obtained from ATCC (Rockville, Maryland). The 293T cell line was cells from human embryonal kidney transformed by adenovirus DNA (ATCC CRL-1573). The COS-7 cells were cultured in RPMI1604 medium, and the 293T cells were cultured in opti DMEM medium (Gibco).

Selection of HD and ALCL Tissues

tk;2Tissue sections from 16 patients with ALCL and 21 patients with HD, including 13 nodular sclerosing, 7 mixed cellularity, and 1 lymphocyte-depletion type, were selected. The diagnosis of HD was confirmed by the characteristic histopathology of the involved lymph nodes and the characteristic cytologic features of H-RS cells, which were $CD30^+$ and $CD3^-/CD20^-/CD45^-$.

The diagnosis of CD30-positive ALCL was made based on the following criteria: (1) large anaplastic cells with abundant cytoplasm and large irregular nuclei as defined by the updated Kiel classification and the revised European-American lymphoma classification and (2) CD30 expression on nearly all of the neoplastic cells. The immunophenotypes of tumor cells were available in all 16 ALCL cases, 6 of which were of T-cell origin ($CD45RO^+$), 1 of B-cell origin ($CD20^+$), and 9 of null-cell origin. The presence of *t*(2;5) was confirmed in seven patients by genetic translocation of the *NPM* gene on chromosome 5 by Southern blot analysis, RT-PCR, or cytogenetic analysis (Shiota et al, 1995).

Eleven of the 16 patients who met these criteria received combination chemotherapy as their initial treatment. Six patients were treated with 13-*cis* retinoic acid as primary or salvage treatment (Chou et al, 1996). The median duration of complete remission and of overall survival and the difference in survival among stages were not evaluated because of the short clinical follow-up period for our patients.

Isolation of H-RS Cells or ALCL Cells from Patient Specimens

H-RS cells ($CD15^+/CD45^-/CD45RO^-/EMA^-$) were obtained from three surgical specimens, including two lymph nodes and a spleen, from three different patients. ALCL cells ($CD15^-/CD20^-/CD45RO^-$) were obtained from two surgically resected lymph nodes from two different patients. These specimens were selected because of extensive involvement with the disease and because ample numbers of lymphoma cells were present; ie, H-RS cells >15% and ALCL cells >80%. The sterile tumor tissues were minced and filtered through nylon mesh, and cells were collected by Ficoll-Hypaque gradient centrifugation. The pelleted cells (un-enriched samples) were resus-

pended in RPMI 1640 medium containing 10% fetal calf serum.

Enrichment and Culture of H-RS Cells

H-RS cells were enriched after complement-mediated cytolysis of contaminating T cells, B cells, or monocytes. The effectiveness of H-RS cell enrichment has been discussed in detail previously (Hsu et al, 1987). The cells were lysed by being subjected to two treatments with MAb cocktails containing anti-Leu 1 (CD5), -Leu 2a (CD8), -Leu 3a (CD4), -Leu M5 (CD11c), and anti-IgM, followed by fresh rabbit complement. These antibodies were purchased from Becton-Dickinson (San Jose, California). The lysed cells were removed on Ficoll-Hypaque gradients. The cells were recovered with viabilities of 85–95%.

The presence of H-RS cells in the enriched suspension was confirmed by positive staining with anti-CD30. Contaminating T cells, B cells, and monocytes, which were present at less than 5%, were detected by staining with anti-CD3, anti-CD20, and anti-CD11b. The enriched H-RS cells were then cultured at 1 to 5 $\times$ 10^5 cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 mM 2-mercaptoethanol, and 10 μg/ml penicillin/streptomycin at 37° C in a humidified, 5% CO_2 atmosphere. The cultures could be maintained for up to 7 days. Cell viability ranged from 70–80%.

Effects of CD30L on Cultured H-RS or ALCL Cells

To minimize binding between soluble CD30 and CD30L, we washed the cells thoroughly and added fresh culture medium before CD30L-expressing COS-7 or 293T cells, or anti-CD30 (HeFi-1, functions as CD30L) were added. Cells were cultured at 1 $\times$ 10^5 cells/ml on 96-well microplates (each well 200 μl). Anti-CD30 (HeFi-1 or Ber-H2) was added at a concentration of 10 μg/ml. The CD30L-expressing cells were added to the culture in a 1:1 to 1:5 ratio (CD30L-transfected cells:H-RS or ALCL cells) and maintained for 48 hours. The CD30L-induced proliferation or growth inhibition was evaluated from thymidine or BrdUrd uptake, a colorimetric nonradioactive MTS/PMS assay, the number of cells in culture, and the viability of the cells.

For thymidine uptake, the cells were pulsed with 0.1 μCi/well of [^{3}H]-thymidine ([^{3}H]-TdR, 25 Ci/mmole; Amersham, Arlington Heights, Illinois) for an additional 2 hours. The cells were harvested onto glass fiber filters, and the incorporated cpm (mean cpm $\pm$ SD) were assessed with a gamma counter (LKB Instruments Inc, Houston, Texas). All tests were performed in triplicate and were repeated three times. As control for the specificity of CD30L activity, we treated vector-transformed COS-7 cells or nonimmune mouse ascites at the same concentration.

For colorimetric nonradioactive MTS/PMS assay, the cells were cultured in the presence of 20 μl of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) and PMS (phenazine methosulphate) solution (Cell Titer96 Aqueous Non-radioactive Cell Proliferation Assay kit, Promega, Madison, Wisconsin) for 3 additional hours. The ODs were measured in an ELISA reader at a wavelength of 490 nm.

For BrdUrd uptake, the cells were cultured in the presence of BrdUrd (1×10^{-6} M) for 2 additional hours before they were harvested. The cells were washed with Tris-buffered saline (0.1 M, pH 7.6), prepared as cytospin smears, and examined for uptake of BrdUrd by the nuclei.

Production of CD30L by Cultured H-RS or ALCL Cells

The expression of CD30L mRNA in long-term cultured CD30-positive cells was examined by RT-PCR. RNAs from cultured cells and from PHA-activated normal T cells were prepared and reverse-transcribed into cDNA. Briefly, 12 μl of total RNA (1–5 μg) was mixed with 1 μl of oligo-dT, heated to 70° C for 10 minutes, and then quick-chilled to 0° C. Two microliters of 10$\times$ synthesis buffer (200 mM Tris-HCl, pH 8.4; 500 mM KCl; 25 mM $MgCl_2$; 1 mg/ml BSA), 1 μl of 10 mM dNTP mix, 2 μl of 0.1 M DTT, and 1 μl of RNasin (20 U, Promega) were added with 1 μl of Super Script reverse transcriptase (200 U/ml, BRL, Life Technologies Inc, Gaithersburg, Maryland). The reaction mixture was incubated at 42° C for 60 minutes, then heated at 90° C for 5 minutes for inactivation of the enzyme. Finally, 1 μl of RNase H (2 U/ml) was added to the mixture, which was incubated at 37° C for 20 minutes.

For PCR, we prepared a reaction mixture containing 10$\times$ synthesis buffer, 8 μl; sterile double-distilled water, 68 μl; 10 mM amplification primers, 1 μl each; and 2–5 U/l *Taq* DNA polymerase (BRL), 1 μl. Thirty-five rounds of PCR were performed by cycling at 94° C for 20 seconds, 55° C for 20 seconds, and 72° C for 30 seconds in a programmable thermocycler (USA/Scientific Plastics, Ocala, Florida).

For visualization, a portion of the PCR mixture (6 μl) was added to 1 μl of stop buffer (500 μl glycerol, 200 μl 0.5 M EDTA, 146 μl 1% bromophenol blue, 35 μl 20% SDS, 150 μl 1 M Tris-HCl, pH 7.8, and 30 μl distilled water), and electrophoresis was performed in a 75 V constant-voltage field in 3% NuSieve GTG/1% Sea Kem ME agarose. The specificities of the amplified products obtained with the CD30L primers were confirmed by sequencing.

The oligonucleotide primers for CD30L were prepared based on the published sequences (10, 18). The human β-actin primers (as controls) were 5′-CTCTTCCAGCCTTCCTTCCTG-3′ (sense) and 5′-GAAGCATTTGCGGTGGACGAT-3′ (antisense). These primers were synthesized by the solid-phase β-cyanoethyl phosphoramidite method on an automated DNA synthesizer (Cyclone Plus, MilliGen/Biosearch, Novato, California) and were purified by electrophoresis on polyacrylamide gels.

Expression of CD30L by H-RS Cells or ALCL in Tissue Sections

An avidin-biotin-peroxidase complex method was used for detecting the expression of CD30L in

formalin-fixed, paraffin-embedded tissue sections (Hsu et al, 1981). The antibody was added at 2 μg/ml; this was followed by addition of biotin-labeled rabbit antigoat Ig. After extensive washing, the sections were incubated with avidin-biotin-peroxidase complex and then developed in DAB-H_2O_2 solution. As control for the staining specificity, we omitted the primary antibody or used the preabsorbed antibody. Absorption was performed by incubation of anti-CD30L with the peptide that was used for generation of antibody. The sections were counterstained with hematoxylin, dehydrated, and cleared as in routine processing.

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