Functional expression of receptor activator of nuclear factor κB in Hodgkin disease cell lines

Paolo Fiumara, Virginia Snell, Yang Li, Asok Mukhopadhyay, Mamoun Younes, Ann Marie Gillenwater, Fernando Cabanillas, Bharat B. Aggarwal, and Anas Younes

The malignant Hodgkin and Reed-Sternberg (H/RS) cells of Hodgkin disease (HD) express several members of the tumor necrosis factor (TNF) receptor family, including CD30 and CD40, and secrete several cytokines and chemokines. However, little is known about how regulates cytokine and chemokine secretion in H/RS cells. Although H/RS cells are predominately of B-cell origin, they frequently share phenotypic and functional features with dendritic cells (DCs). Previous studies reported that receptor activator of nuclear factor κB (NF-κB) (RANK), a member of the TNF receptor family, is expressed on DCs, and that RANK ligand (RANKL) enhances DC survival and induces them to secrete cytokines. This study reports that, similar to DCs, cultured H/RS cells expressed RANK. However, unlike DCs, H/RS cells also expressed RANKL. Soluble RANKL activated NF-κB and induced messenger RNA expression of interferon-γ, interleukin-8 (IL-8), IL-13, IL-9, IL-15, and RANTES. In addition to the receptors for IL-9, IL-13, IL-15, and CCR4, RANKL increased IL-8 and IL-13 levels in the supernatants of H/RS cell lines, an effect that was blocked by soluble RANK. Furthermore, soluble RANK decreased the basal level of IL-8 in one cell line, suggesting that IL-8 was induced by an autocrine RANKL/RANK loop. RANKL had no effect on H/RS cell survival in culture, and it did not modulate the expression of bcl-2, bcl-xL, bax, or inhibitors of apoptosis proteins. These data provide evidence of further functional similarities between DCs and H/RS cells. The coexpression of RANK and RANKL in H/RS cells suggests that they may regulate cytokine and chemokine secretion in H/RS cells by an autocrine mechanism. (Blood. 2001;98:2784-2790)

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malignant cells are unknown. Because of the similarities between H/RS cells and DCs, and because H/RS cells secrete a wide variety of cytokines that have been shown to be induced by RANKL, we examined the expression of RANK in cultured H/RS cells and determined the function of this expression.

Materials and methods

Cell lines and reagents

The human H/RS-derived cell lines KM-H2, HDLM-2, L-428, and HD-MYZ were obtained from the German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures (Braunschweig, Germany). The phenotype and genotype of these cell lines have been previously published.31 All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, l-glutamine, and penicillin/streptomycin (Gibco BRL, Gaithersburg, MD) in a humid environment of 5% CO2 at 37°C.

Recombinant human RANKL, CD40 ligand (CD40L, CD154), and activating antibody to human Fas (CH-11), soluble CD30, soluble RANK, and soluble OPG were from Alexis (San Diego, CA). Recombinant human TRAIL trimer (leucine zipper) and activating antibody to human CD30 receptor (M44)32 were kindly provided by Dr Raymond Goodwin (Immunex, Seattle, WA). Antibodies to RANK, RANKL, and OPG were from Ingexen (San Diego, CA); to Bcl-x, Bcl-2, Bax, and cellular inhibitors of apoptosis proteins 2 (cIAP2) were from Santa Cruz Biotechnology (Santa Cruz, CA); to FLIP and cIAP1, survivin, and NAIP were from R & D Systems (Minneapolis, MN); to XIAP was from Transduction Laboratories (San Diego, CA); and to β-actin was from Sigma Chemicals (St Louis, MO).

Assessment of cell viability

Cells were cultured in 24-well plates in a volume of 500 μL at 5 × 105 cells/mL for all cell lines. Cell viability was assessed with a nonradioactive cell proliferation MTS assay using CellTiter96 Aqueous One Reagent (Promega, Madison, WI), according to the manufacturer’s instructions.33 In this assay, formazan absorbance was measured at 490 nm on a μQuant plate reader equipped with KC4 software (Biotek Instruments, Winooski, VT). Each measurement was made in triplicate and the mean value was determined.

Flow cytometry

Cells were stained with fluorescein isothiocyanate (FITC)– or phycoerythrin (PE)–conjugated antibodies to CD30, CD40, CD95 (Fas), CD154 (CD40L), B7.1, B7.2, or isotype-matched control antibodies (all from Pharmingen, San Diego, CA) as previously described.34,35 Data were collected on a Becton Dickinson FACScan flow cytometer and analyzed by WinMDI 2.8 software (Joseph Trotter, Scripps, San Diego, CA).

Western blot analysis

Cellular protein was extracted by incubation in RIPA buffer (Roche Molecular Biochemicals, Indianapolis, IN) for 15 minutes at 4°C and then centrifuged to remove cellular debris. The protein in the resulting supernatant was quantified by the bicinchoninic acid (BCA) method according to the manufacturer’s instructions (Pierce, Rockford, IL), diluted 1:1 in protein-loading buffer (0.25 M Tris-HCl, 2% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 1% glycerol, and 0.2 mg/mL bromophenol blue), and boiled for 30 minutes. A total of 30 μg protein was loaded onto 12% Tris-HCl SDS–polyacrylamide gel electrophoresis (SDS-PAGE) Ready Gels (Bio-Rad, Hercules, CA), transferred to a nitrocellulose transfer membrane (Osmonics, Minnetonka, MN), and detected using ECL-Plus (Amersham, Buckinghamshire, United Kingdom).

Electromobility shift assay and antibody supershift assay

Electromobility shift assays (EMSAs) were performed to determine the activation and nuclear translocation of NF-κB as previously described, with minor modifications.36 Briefly, 4 μg nuclear protein extract was incubated with 16 fmol of a 32P-labeled 45-mer double-stranded DNA oligonucleotide derived from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAGGGACTTTCCGCTGGGACTTTCCAGGGAGGC-GTGG-3′) (underlined areas indicate NF-κB binding sites) for 30 minutes at 37°C. The resulting complex was resolved from free oligonucleotide by electrophoresis on 6.6% native polyacrylamide gels. To determine the specificity of NF-κB, antibody supershift assays were performed as previously described.31 Briefly, nuclear protein extract was incubated with antibodies against different NF-κB subunits (p50 and p65), control antibody (cyclin-D1), preimmune serum (PBS), unlabeled oligonucleotide, and mutant oligonucleotide: 5′-TTGTTACAACTCACTTTCCGCTGGCTC- CACTTTCCAGGGAGGCGTG-3′) for 30 minutes and then assessed for NF-κB by EMSA.

RNSa protection assay

The mRNA expression of cytokines and their receptors plus chemokines and their receptors was determined by RNase protection assay using RibobQuant kits from Pharmingen. Briefly, cell lines (0.5 × 106/mL) were incubated with RPMI or RANKL (1 μg/mL) for 24 hours. Total RNA was isolated from 1 × 107 cells using the guanidium-isothiocyanate method. Ten micrograms of RNA from each sample was hybridized to a 32P-labeled antisense probe set (hCR-1, hCR-5, hCK-1, hCK-5, hCR-6, h-AP0-3d) and digested with RNase and T1 nuclease. The protected probe fragments were resolved on 5% polyacrylamide gels according to the manufacturer’s instructions. Band intensity was quantified by National Institutes of Health image software (version 1.6.1) and normalized to the intensity of GAPDH probe.

Immunohistochemistry

Paraffin-embedded lymph node biopsy sections from patients with nodular sclerosis HD were immunostained using a Techmate 1000 automatic immunostainer (Ventana, Tucson, AZ) as described previously.38 Briefly, sections were deparaffinized in xylene, rehydrated with decreasing concentrations of alcohol and finally phosphate-buffered saline (PBS), and subjected to steam-heat epitope retrieval in 10 mM citrate buffer (pH 6.0) for 30 minutes in a commercially available vegetable steamer. The sections were then rinsed in distilled water, washed in PBS for 5 minutes, and incubated for 15 minutes with DAKO protein block (DAKO, Carpenteria, CA). Next, they were incubated for 2 hours with anti-RANK monoclonal antibody from Alexis, diluted 1:500 in 0.1% bovine serum albumin/PBS. (Results were confirmed using a polyclonal antibody from Santa Cruz diluted at 1:50.) Then sections were washed, and bound antibodies were detected using an LSAB2 peroxidase kit (primary rabbit/mouse; DAKO) with diaminobenzidine as chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were biopsy sections that were immunostained with either nonreactive mouse IgG diluted to the same concentration as the anti-RANK antibody or with no antibody. Results of CD30, CD15, CD20, and LMP-1 expression in primary H/RS cells were available from pre-existing diagnostic reports in 7 patients.

Enzyme-linked immunosorbent assay

Human IL-8 and IL-13 levels were determined in H/RS cell supernatants, after incubation with RANKL (2 μg/mL), soluble RANKL (5 μg/mL), or both. Incubations were performed for 24 to 48 hours. Commercially available enzyme-linked immunosorbent assay (ELISA) kits from R & D Systems were used according to the manufacturer’s instructions. The lower limit of sensitivity of this assay is 10 pg/mL for IL-8 and 32 pg/mL for IL-13. The results were read at an optical density of 450 nm using a Vmax ELISA reader (Molecular Devices, Menlo Park, CA). Measurements were done in triplicate and results are reported as the mean ± SD.
Functional expression of RANK in H/RS cells

We examined the expression of RANKL and its 2 receptors, RANK and OPG, in 4 well-characterized H/RS cell lines using Western blot analysis. The RANK protein was most prominently expressed in HDLM-2 and L-428. The remaining 2 cell lines also expressed RANKL, but at very low levels. All cell lines expressed RANK and RANKL at the mRNA level (data not shown). RANKL and OPG were expressed in all 4 HD cell lines (Figure 1A). Results were then compared with those of other cell lines of hematopoietic origin (Table 1). In these cell lines, RANK was most prominently expressed by the multiple myeloma cell line 8226 and was weakly expressed by the T lymphoblastic Jurkat cell line and the anaplastic large cell lymphoma DHL-1 cell line. As shown in Table 1, the phenotype of the cell line (B or T), the expression of CD30, CD40, or Epstein-Barr virus (EBV) did not correlate with RANK expression. All these cell lines expressed OPG, whereas RANKL was expressed in 7 of the 9 cell lines tested (Table 1).

Expression of RANK was also evaluated in primary lymphoid tumors (Figures 1B and 2). Using Western blot analysis, RANK expression could not be detected in 5 primary non-Hodgkin lymphoma specimens, regardless of their phenotype (Figure 1B). In contrast, RANKL and OPG were variably expressed in all these specimens. Using immunohistochemistry, RANK was detected in primary H/RS cells in 10 lymph node sections (Figure 2A,B), but it was rarely, weakly expressed in sections from small lymphocytic lymphoma (Figure 2C) or from a benign hyperplastic lymph node (Figure 2D). Within each lymph node section, an average of 75% of H/RS cells (range 10% to 75%) expressed RANK (Table 2), which showed a predominantly cytoplasmic staining pattern (Figure 2), but it was rarely, weakly expressed in the benign infiltrating cells (Figure 2A,B). There was no difference in the pattern or frequency of RANK expression in cases of nodular sclerosis or mixed cellularity (Table 2). C30, CD15, CD20, or LMP-1 expression data were available on 7 of the 10 primary HD sections (3 nodular sclerosis and 4 mixed cellularity), and no correlation could be found between RANK expression and the expression of these antigens (Table 2).

RANK was previously reported to activate NF-κB in different cell systems.13,39 To investigate whether the expression of RANK in the H/RS cell lines was functional, we incubated these cell lines with RANKL (1 μg/mL) and studied NF-κB activation by EMSA. RANKL activation of NF-κB was most prominent in HDLM-2 cells, because it could be detected within 10 minutes of stimulation and peaked at 60 minutes of stimulation. Activation of NF-κB was less prominent in HD-MYZ and L-428 cells and was not observed in the KM-H2 cells (Figure 3).

Effect of RANKL on cultured H/RS cell survival in vitro

RANKL has been reported to be a survival factor for DCs.23 To investigate whether RANKL plays a similar role in H/RS cells, we incubated these 4 cell lines with increasing concentrations of RANKL (0-1000 ng/mL) for 24 to 72 hours. Cell viability and proliferation were determined using the MTS assay. RANKL had no significant effect on the survival or proliferation of any of the H/RS cell lines in vitro (Figure 4A). Because these cell lines coexpressed RANKL and RANK, we hypothesized that these cells may have been maximally stimulated with endogenous RANKL through an autocrine survival loop. Therefore, we reasoned that if we interrupted this loop, we might decrease cell survival. To test this hypothesis, we incubated H/RS cell lines with increasing concentrations of soluble RANK and

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Table 1. Expression of RANK, RANKL, and OPG in HD-derived cell lines and other hematopoietic cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phenotype/cell lineage</th>
<th>Expression of</th>
<th>RANK</th>
<th>RANKL</th>
<th>OPG</th>
<th>CD30</th>
<th>CD40</th>
<th>EBV</th>
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<tbody>
<tr>
<td>HD-MYZ</td>
<td>Monocytoid</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HDLM-2</td>
<td>T</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-428</td>
<td>B/T</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>KM-H2</td>
<td>B</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
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<td></td>
<td>-/+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SUP-M2</td>
<td>T</td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8226</td>
<td>B</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

UK indicates unknown.
soluble OPG. H/RS cells incubated with soluble CD30 were used as a control. At concentrations ranging from 5 to 500 ng/mL neither soluble RANK nor OPG had an effect on H/RS cell survival in vitro (Figure 4B).

The effect of RANKL on intracellular proteins that influence cell survival was subsequently investigated in the H/RS cell lines. Cells were incubated with RANKL (1 μg/mL) or medium for 24 or 48 hours and the levels of intracellular proteins were measured by Western blot analysis. RANKL had no effect on the expression of Bcl-xL, Bax, or Bcl-2 proteins (data not shown). Furthermore, RANKL had no effect on the antiapoptotic protein FLICE-inhibiting protein (cFLIP) or on any of the inhibitors of apoptosis proteins (IAPs) (data not shown).

Although RANKL had no significant effect on the survival of cultured H/RS cells in vitro, we examined whether RANKL can modulate the apoptotic effect induced by chemotherapy, TRAIL, or Fas ligand. Cells were incubated with doxorubicin (0.5 μg/mL), RANK (1 μg/mL), or both for 24 or 48 hours and the viable cell number was determined using the MTS assay. Doxorubicin was effective in killing L-428, HDLM-2, and KM-H2 cells (data not shown). The combination of doxorubicin plus RANKL was not different from doxorubicin alone, indicating that RANKL could not inhibit doxorubicin-induced cell death in these H/RS cell lines. Similarly, RANKL had no effect on FasL- or TRAIL-induced cell death in the H/RS-sensitive cell lines (data not shown).

**Effect of RANKL on cell surface protein expression**

The H/RS cells frequently express TNF receptor family members, including CD30, CD40, and CD95.40 In addition, H/RS cells frequently express the costimulatory molecules B7.1 and B7.2. To determine whether RANKL is involved in regulating the expression of these proteins, cultured H/RS cell lines were incubated with

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**Table 2. Expression of RANK and other antigens in primary H/RS cells as determined by immunohistochemistry**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Histologic type</th>
<th>Results of histochemical staining of primary H/RS cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RANK (percent of H/RS cells)</td>
<td>CD30</td>
</tr>
<tr>
<td>1</td>
<td>NS &gt; 75</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>NS &gt; 75</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>MC 50-75</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>NS &gt; 75</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>MC 50-75</td>
<td>+</td>
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<tr>
<td>6</td>
<td>MC &lt; 10</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>MC &gt; 75</td>
<td>+</td>
</tr>
</tbody>
</table>

NS indicates nodular sclerosis; MC, mixed cellularity; ND, not done.

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**Figure 2. Immunohistochemical staining of RANK protein in primary lymphoid tumors.** RANK was highly expressed in H/RS cells of a lymph node involved with nodular sclerosis (A) and mixed cellularity (B). The surrounding infiltrating cells rarely, but weakly, expressed RANK. Section from a lymph node involved with B-cell lymphocytic lymphoma (C) and from a benign hyperplastic lymph node (D) showing that RANK was rarely, but weakly expressed in these sections.
Table 3. Effect of RANKL on cytokine and chemokine mRNA expression in H/RS cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IFN-γ</th>
<th>IL-8</th>
<th>RANTES</th>
<th>CCR-4</th>
<th>IL-9</th>
<th>IL-9Rα</th>
<th>IL-15</th>
<th>IL-15Rβ</th>
<th>IL-13</th>
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<tr>
<td>HDLM-2</td>
<td>2</td>
<td>1.5</td>
<td>3</td>
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<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>HD-MYZ</td>
<td>4</td>
<td>3</td>
<td>4.6</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-428</td>
<td></td>
<td></td>
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<td>KMH-2</td>
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</table>

Cells were incubated with RPMI or RANKL (1 μg/mL) for 6 to 24 hours before mRNA levels were determined using the RNase protection assay.
DCs, activated T cells, and osteoclasts.23) Except for activated T thymus, whereas RANK protein is predominantly expressed by skeletal muscles, colon and intestines, adrenal glands, and the observation that RANK

...tic germinal center cells, but the physiologic role of this...z

...tion of IL-1, IL-6, IL-12, and IL-15. 21) In our study, different...line predominantly induced the expression of IL-9 and CCR4, and to a lesser extent IFN-γ, IL-15, IL-13, and RANTES. RANK activation in the L-428 cell line induced IFN-γ, IL-13, and RANTES expression, whereas RANKL up-regulated IL-15 expression in the KMH-2 cell line. It is possible that RANK activation is involved in regulating other cytokines and chemokines that were not tested in this study. Complementary DNA microarray experiments will better define the outcome of RANK activation in these cell lines.

The expressions of IL-13 and IL-13 receptor have been recently reported in cultured and primary H/RS cells and play a role in the survival of H/RS cells.3,42) Therefore, the ability of RANK to up-regulate both IL-13 and IL-13 receptor suggests that RANK may indirectly have a role in the growth regulation of H/RS cells. RANK may also play an important role in regulating the cellular infiltrate surrounding H/RS cells by regulating the expression of critical chemokines such as IL-8.43

In addition to the reported similarities between H/RS cells and DCs, we found new common features between these 2 cell types. Both cell types express RANK, and RANK activation induces the expression of several cytokines and chemokines. However, several important differences between H/RS cells and DCs were also observed in this study. First, DCs do not express RANKL. Second, unlike DCs, whereas RANK activation provides survival signals by up-regulating Bcl-xL,44 we found no role for RANK in regulating the survival or proliferation of the tested H/RS cell lines. RANK activation in H/RS cells did not regulate the expression of several intracellular proteins that are known to be involved in regulating cell life and death, including Bcl-xL, Bax, Bcl-2, cFLIP, and IAPs. Whether RANKL may provide survival signals to primary H/RS cells is currently unknown. In B cells, IL-13 and CD40L can inhibit apoptosis by up-regulating Bcl-xL.45) It is unknown whether RANKL may act synergistically with other survival factors such as IL-13, CD40L, or CD30L. Third, CD40L was reported to up-regulate RANK in DCs,13 but failed to do so in H/RS cells.

In normal tissues, RANKL mRNA is detected in the thymus, lymph nodes, and resting CD4+ and CD8+ T lymphocytes. RANKL protein is expressed by osteoblasts, bone stromal cells, and activated T lymphocytes. In this study, all the H/RS cell lines expressed RANK regardless of their phenotype (B, T, or monocyte-oid), in addition to 2 T-cell lines (Jurkat and SUP-M2) and a multiple myeloma B-cell line (8226). Interestingly, RANKL was also expressed by primary B and T lymphomas. The role of RANKL in these B- and T-cell primary lymphoma tumors remains unclear.

The expression of RANK and RANKL in H/RS cells suggests that they may regulate cytokine and chemokine expression by an autocrine loop mechanism. In short-term culture, interrupting this autocrine loop by soluble RANK had no effect on H/RS cell survival. It is not known whether interrupting this autocrine loop may decrease the cellular infiltrate around primary H/RS cells. Although RANKL did not enhance HD cell survival, down-regulation of certain cytokines and chemokines may indirectly influence H/RS cell survival by decreasing the cellular infiltrate that may provide survival signals.

In conclusion, our study shows for the first time that RANK and RANKL are functionally expressed in H/RS cell lines and that RANK is expressed in primary H/RS cells. The expression of RANK and RANKL is likely to be involved in regulating the cellular infiltrate and cytokine and chemokine secretion in HD.

Figure 6. RANKL induces IL-13 and IL-8 secretion in H/RS cells. (A) HDLM-2 cells were incubated with RPMI, RANKL, soluble RANK receptor, or both RANKL and RANK for 48 hours. Supernatants were collected and assayed (without dilution) for IL-13 by ELISA. RANKL increased IL-13 from 95 pg/mL to 340 pg/mL. (B) A similar experiment was performed using the HD-MYZ cell line. The level of IL-8 was measured after 24 hours in culture (supernatants were diluted at 1:20). In this cell line, soluble RANK decreased the baseline level of IL-8 and blocked the effect of exogenous RANKL.
References

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