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Identification of Rat OX40 Ligand by Molecular Cloning

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OX40 (CD134) is a member of the tumor necrosis factor (TNF) receptor superfamily first identified as a rat T cell activation marker. In the present study, we identified the rat ligand for OX40 (OX40L) by molecular cloning. Rat OX40L cDNA was cloned from a HTLV-1-transformed rat T cell line by cross-hybridization with mouse OX40L cDNA. The predicted rat OX40L polypeptide is composed of 199 amino acids, showing 80.9 and 43.3% homology to mouse and human OX40L, respectively. Expression of rat OX40L mRNA was found in HTLV-1-transformed rat T cell lines. Expression of OX40L on the cell surface of these HTLV-1-transformed rat T cell lines was also demonstrated by flow cytometric analysis with a soluble fusion protein composed of the extracellular region of the Fc portion of human IgG (OX40-Ig). To explore the function of rat OX40L, we generated cDNA transfectants stably expressing rat OX40L. The rat OX40L transfectants exhibited a potent costimulatory activity for proliferation and IL-2 production of anti-CD3-stimulated rat T cells. These results indicated that rat OX40L can provide an efficient costimulation for rat T cells and that it may be involved in HTLV-1-associated pathologies in the rat system as has been suggested in the human system.

OX40 (CD134) was originally identified as a cell surface antigen on activated rat $CD4^+$ T cells using MRC OX40 mAb (1). cDNA cloning of rat, mouse, and human OX40 showed that it belongs to the tumor necrosis factor (TNF) receptor superfamily (2-4). A ligand for OX40 (OX40L) has recently been identified in mice and humans, but still not in rats, which is a type II membrane protein belonging to the TNF superfamily (5, 6). Members of the TNF/TNF receptor superfamily have been shown to play critical roles in regulating cellular activation, differentiation, and apoptosis (7-9).

Expression and function of the OX40-OX40L molecules have been studied in the mouse and human systems. It has been shown that the expression of OX40 is restricted to activated T cells in rats, humans, and mice (1, 3, 10). The expression of OX40L has been found on activated mouse B cells (3, 11), human dendritic cells (DC) (12), and human vascular endothelial cells (13). *In vitro* studies using mouse and human OX40L cDNA transfectants have shown that OX40L provides a co-stimulatory signal to T cells, resulting in increased proliferation and cytokine production (1, 5, 6). On the other hand, OX40L expressed on activated B cells has been shown to transmit a signal that enhances proliferation and secretion of immunoglobulin upon crosslinking by OX40 (11). In addition, a resent study demonstrated that ligation of OX40L on human DC enhanced their maturation and production of cytokines (12). Collectively, these *in vitro* studies suggested that the OX40/OX40L system may play an important role in T-B and T-DC interactions. To support this notion, Stüber and Strober have reported that administration of anti-OX40 antibody inhibited the T cell-dependent humoral immune response in mice, possibly by interrupting the OX40/OX40L interaction (10). Besides such physiological functions, OX40 has been suggested to be preferentially expressed on pathogenic T cells in experimental autoimmune encephalomyelitis (EAE) in rats, and acute graft-versus-host disease (GVHD) and rhematoid arthritis (RA) in humans (14-16). Furthermore, it has been shown that both OX40 and OX40L are expressed at high levels on HTLV-1-transformed human T cells possibly due to transactivation by Tax, suggesting that the OX40/OX40L system may be involved in growth and malignant transformation of HTLV-1-infected T cells (17-21). However, the possible contribution of the OX40/OX40L system to these diseases remains to be verified in appropriate animal models.

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Abbreviations used: OX40L, OX40 ligand; HTLV-1, human T cell leukemia virus type I; DC, dendritic cells; mAb, monoclonal antibody; EAE, experimental autoimmune encephalomyelitis; GVHD, acute graft-versus-host disease; RA, rhematoid arthritis; EAU, experimental autoimmune uveoretinitis; PHA, phytohemagglutinin; PMA, phorbol myristate acetate.

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                                        GGCACGAGCTTCAATTGCCTTTTGTCTCCGGTTCTGGGA   39
CCTTTGTCTTCTGACCCACAGGCTTGGCTTCACCCTTGTCTTCTCCTTTGTGGTGAAGAG   99
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rat OX40L       1 MEGEGVQPPDENLENGSRPRFKWKKVLRLVVSGIKAAGLLLCVVYVCLQFSS-SPAKDSP   59
mouse OX40L     1 MEGEGVQPLDENLENGSRPRFKWKKTLRLVVSGIKCAGMLLCFIYVCLQLSS-SPAKDPP   59
human OX40L     1 ME-R-VQPLEENVGNAARPRFERNKLL-LVASVIQGLGLLLCFTYICLHFSALQVSHRYP   57

rat OX40L      60 -IQRLRAPVTGCEGGRLFIGTSKNEYETMEVQNNSVIINCDGLYLIHLKGSFFQEVKINL  118
mouse OX40L    60 -IQRLRGAVTRCEDGQLFISSYKNEYQTMEVQNNSVVIKCDGLYIIYLKGSFFQEVKIDL  118
human OX40L    58 RIQSIKVQFTEYKKEKGFILTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVNISL  117

rat OX40L     119 HFRKDRSPIFVPMLNNGQRVVFTVVTSLAFKDEVYLTVNASDTLCEHLQINDGELIIVQL  178
mouse OX40L   119 HFREDHNPISIPMLNDGRRIVFTVVASLAFKDKVYLTVNAPDTLCEHLQINDGELIVVQL  178
human OX40L   118 HYQKDEEPL-FQ-LKKVRSVNSLMVASLTYKDKVYLNVTTDNTSLDDFHVNGGELILIHQ  175

rat OX40L     179 TPNGYCAPERPYSSTVNQVPL                                       199
mouse OX40L   179 TP-GYCAPEGSYHSTVNQVPL                                       198
human OX40L   176 NPGEFCVL-------------                                       183
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To further explore the physiological and pathological functions of OX40L, the rat system has some advantages over the human and mouse system. Superior models of autoimmune diseases, such as collagen-induced arthritis, experimental autoimmune uveoretinitis (EAU) and EAE have been developed (22-24). Some models of human diseases, such as HTLV-1-associated pathologies (25), are unique in the rat system. In order to facilitate the studies in the rat system, we now identified rat OX40L by molecular cloning and characterized its expression and function. Aberrant expression of OX40L in HTLV-1-transformed rat T cell lines was noted.

MATERIALS AND METHODS

Animals and cells. Six-week-old female SD rats were purchased from Charles River Japan (Atugi, Japan). HTLV-1-transformed rat T cell lines (F344-S1, ACI-S1, WKA-S1, and Lewis-S1) (26) were provided by Dr. T. Yoshiki (Hokkaido University, Sapporo, Japan). A rat thymic lymphoma cell line (FTL13) (27) was provided by Dr. T. Uede (Hokkaido University). A rat macrophage cell line (WRT7/P2) (28) was provided by Dr. M. Hosokawa (Hokkaido University). A murine myeloma P3U1 (P3X63Ag8U.1) was purchased from ATCC (Rockville, MD). These cells were cultured in RPMI1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1mM sodium pyruvate, 0.1mg/ml penicillin and streptomycin, and 50 μM β-mercaptoethanol. A normal rat kidney cell line (NRK-52E) was kindly provided by Dr. T. Otsuka (Institute of Cytosignal Research, Inc., Tokyo, Japan) and was cultured in DMEM containing 10% FCS, 10 mM HEPES, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1mg/ml penicillin and streptomycin, and 50 μM β-mercaptoethanol.

cDNA cloning. A cDNA library was constructed from F344-S1 mRNA. Total RNA was extracted from F344-S1 cells using Trizol (Gibco BRL, Grand Island, NY) and poly(A) RNA was purified by using QuickPrep mRNA purification kit (Pharmacia Biotech, Oppsala, Sweden) according to the manufacturer's instructions. cDNA synthesis was performed using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). cDNA of 1-3kb was isolated by Sepharose CL-2B gel filtration and then ligated to the Uni-ZAP XR vector. Screening was performed by cross-hybridization with a ^{32}P-labeled mouse OX40L cDNA fragment (nucleotide 123-739) (5) which were prepared by RT-PCR from a mouse B cell lymphoma (BCL1-B20) mRNA. The probe was labeled with [α-^{32}P]dCTP using a random primed labeling kit (Amersham, Buckinghamshire, UK) and hybridized in 4×SSC/0.5% SDS, 5×Denhardt's solution and 0.1mg/ml of salmon sperm DNA at 65°C. The blots were washed to a final stringency of 0.5×SSC/0.1% SDS at 65°C and exposed for autoradiography.

DNA sequence analysis. Rat OX40L cDNA in Uni-ZAP XR vector was subcloned into pBluescript plasmid vector by an *in vivo* excision protocol. The cDNA insert was sequenced on both strands using synthetic oligonucleotide primers and dye-labeled terminator/Taq DNA polymerase on an automated fluorescent DNA sequencer (Perkin Elmer, Foster City, CA). Aligment of protein sequences and estimation of homology were performed using GENETYX program (Software development co., LTD., Tokyo, Japan).

Northern blot analysis. Total cellular RNA was prepared using Trizol and 10μg each was electrophoresed in 1% agarose gel in the presence of formamide. The RNA was transferred to a nylon membrane (Pall, East Hills, NY), and the blot was hybridized with ^{32}P-labeled rat OX40L cDNA at 65°C, washed to a final stringency of 0.5×SSC/0.1% SDS at 65°C, and then exposed for autoradiography.

Construction of OX40-Ig fusion protein. To detect the expression of OX40L by flow cytometry, a fusion protein consisting of the extracellular domain of murine OX40 (amino acids 1-211) (3) and the Fc portion of human IgG1 (OX40-Ig) was constructed. RT-PCR was performed using total RNA from concanavalin A-activated mouse splenocytes. 5′-GACGAATTCATGTATGTGTGGGTTCAGCAGC-3′ and 5′-GACGGATC CCAGGGCCCTCAGGAGTCAC-3′ were used as 5′ and 3′ primers, respectively. The PCR product was cloned into the *Eco* RI and *Bam* HI sites of pBluescript SK(+) which contained the gene encoding Fc portion of human IgG1 (kindly provided by Dr. B. Seed, Harvard Medical School, Boston). The insert encoding OX40-Ig was subcloned into the *Xho* I and *Not* I sites of a mammalian expression vector pMKITneo (kindly provided by Dr. K. Maruyama, Tokyo Medical Dental University, Tokyo). Stable transfectants producing OX40-Ig was generated by electroporation into P3U1 cells and selection with 1mg/ml of geneticin (Sigma, St. Louis, MO) as described previously (29). OX40-Ig fusion protein was purified using protein G-Sepharose column (Pharmacia Biotech) from the culture supernatant.

Flow cytometry. Cells (5×10^5) were first preincubated with normal rat IgG to avoid non-specific binding and then incubated with a saturating amount of OX40-Ig fusion protein. After washing with PBS twice, the cells were incubated with phycoerythrin (PE)-conjugated goat anti-human IgG antibody (Caltag, Burlingame, CA). After washing with PBS twice, the stained cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA) and data were processed by using the CellQuest program (Becton Dickinson).

Transfectants. Rat OX40L cDNA was subcloned into the pMKITneo expression vector and transfected into NRK-52E cells by electroporation. Mock transfected cells were also produced by electroporation of the pMKITneo vector without an insert. After selection by 1mg/ml of geneticin, transfectants stably expressing rat OX40L were identified by staining with OX40-Ig.

T cell proliferation and IL-2 assay. Splenic T cells were prepared from SD rat using a nylon wool column (Wako, Osaka, Japan). The cell preparation contained >80% CD3$^+$ cells as estimated by FACS analysis. Rat OX40L- or mock-transfected NRK-52E cells (rOX40L/NRK, mock/NRK) were irradiated (100 Gy). Splenic T cells (5×10^5/well) were co-cultured with irradiated transfectants ($1\times10^2\sim1\times10^4$) in the presence of 1$\mu$g/ml of anti-CD3 mAb (G4.18, mouse IgG3; PharMingen, San Diego, CA) and 0.5μg/ml of anti-mouse IgG3 mAb (PharMingen) in 96-well flat bottomed culture plates at 37°C for 72h. For T cell proliferation assay, the cultures were pulsed with 0.5 μCi/well of [^{3}H]-thymidine (Amersham) for the last 6 h and harvested using a Micro 96 Harvester (Skatron, Lier, Norway). Incorporated radioactivity was measured in a micro β-counter (Micro Beta Plus, Wallac, Turku, Finland). Secretion of IL-2 by stimulated T cells was assayed using the IL-2-dependent cell line, CTLL-2. Supernatants were incubated for 24 h with 1×10^4 CTLL-2 cells in the presence or absence of 10μg/ml of anti-mouse CD25 mAb (3C7, PharMingen) and 0.5 μCi/well of [^{3}H]-thymidine was added 4 h before cell harvest as indicated above.

FIG. 1. Structure of rat OX40L. (A) Nucleotide and amino acid sequences of rat OX40L. The putative transmembrane domain is denoted by a long underline and two potential N-glycosylation sites in the extracellular domain are shaded. Three potential poly(A) additional signal sequences are indicated by short underlines in the 3′ untranslated region. (B) Compartion of rat, mouse, and human OX40L amino acid sequences. Amino acids identical between rat and either mouse or human are shaded. Gaps in sequence alignment are indicated by dashes. Rat OX40L cDNA sequence data will appear in the GenBank under Accession No. AF037067.

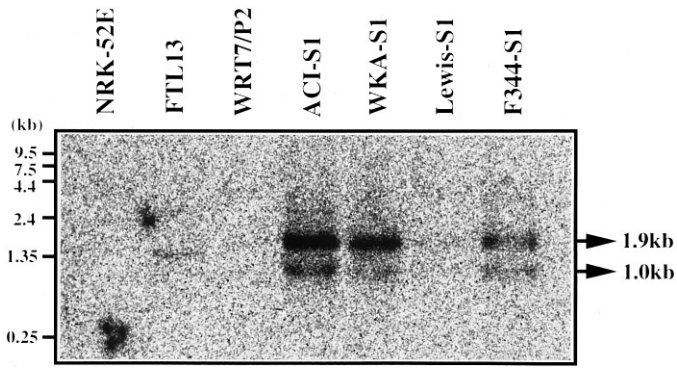

FIG. 2. Expression of rat OX40L mRNA in rat cell lines. Total RNA was isolated from HTLV-1-transformed T cell lines (F344-S1, Lewis-S1, WKA-S1, and ACI-S1), a macrophage cell line (WRT7/P2), a thymic lymphoma cell line (FTL13), and a normal kidney cell line (NRK-52E). Ten microgram each of total RNA was electrophoresed on an agarose gel and transferred onto a nylon membrane. The blot was hybridized with ^{32}P-labeled rat OX40L cDNA probe. The positions of molecular size markers (Gibco-BRL) are shown to the left in kb.

RESULTS AND DISCUSSION

Cloning of Rat OX40 Ligand

In order to identify rat OX40L, we first examined the expression of rat OX40L mRNA in various rat cell lines by Northern blot analysis using mouse OX40L cDNA as the probe. The mRNA expression was observed in an HTLV-1-transformed T cell line, F344-S1 (data not shown). Then, we isolated rat OX40L cDNA from the F344-S1 cDNA library by cross-hybridization with mouse OX40L cDNA probe. As shown in Fig. 1A, rat OX40L cDNA encodes a type II transmembrane protein of 199 amino acids, which is predicted to consist of a cytoplasmic domain of 28 amino acids, a hydrophobic transmembrane domain of 20 amino acids, and an extracellular domain of 151 amino acids. The extracellular domain contains two potential N-linked glycosylation sites. Alignment of rat, mouse, and human OX40L amino acid sequences is shown in Fig. 1B. Rat OX40L is most closely related to mouse OX40L with 80.9% homology and to a lesser extent to human OX40L with 43.3% homology.

Expression of Rat OX40L mRNA

Using rat OX40L cDNA as a probe, we examined the mRNA expression in various rat cell lines. As shown in Fig. 2, the transcripts were abundantly detected as two mRNA bands (~1.0 and ~1.9 kb) in three of four HTLV-1-transformed T cell lines tested (F344-S1, WKA-S1, and ACI-S1). These transcripts were also weakly detected in another HTLV-1-transformed T cell line, Lewis-S1. In contrast, these transcripts were not detected in a rat macrophage cell line (WRT7/P2) and a thymic lymphoma cell line (FTL13). Identity of a ~1.4 kb band detected in FTL13 is presently not clear, but it appears not to encode OX40L since FTL13 does not bind OX40-Ig (see below). It has been reported that both human and mouse OX40L mRNAs were also detected as two bands by Northern blot analysis (5,19). It was supposed that two human OX40L mRNAs were generated by alternative usage of two poly(A) addi-

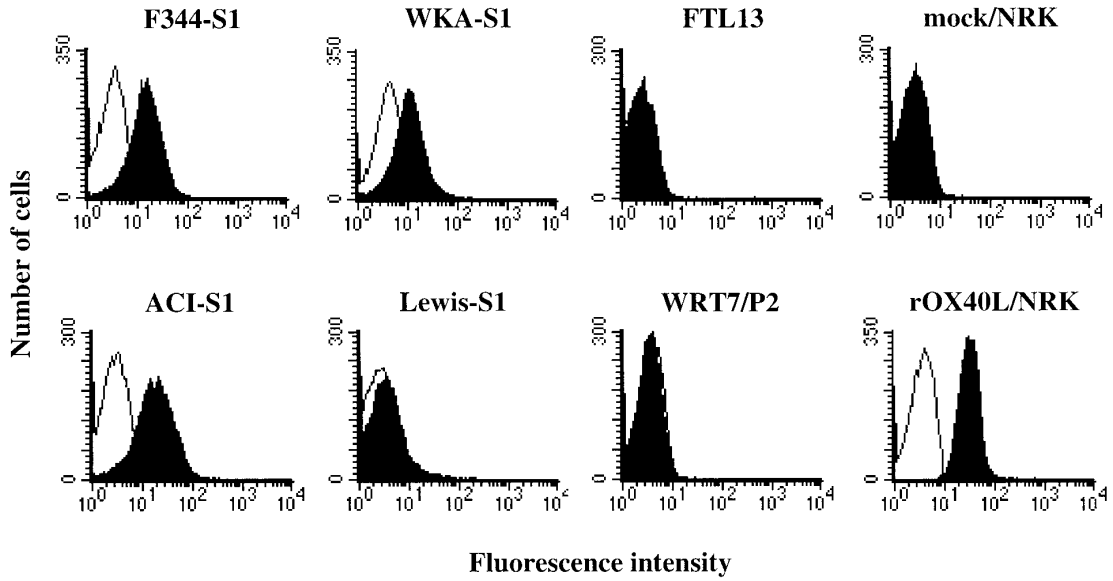

FIG. 3. Cell surface expression of OX40L on rat cell lines. HTLV-1-transformed T cell lines (F344-S1, WKA-S1, ACI-S1, and Lewis-S1), a macrophage cell line (WRT7/P2), a thymic lymphoma cell line (FTL13), and NRK-52E-derived transfectants (rOX40L/NRK and mock/NRK) were stained with OX40-Ig followed by PE-conjugated goat anti-human IgG. The shaded peaks represent staining with OX40-Ig and the blank peaks represent background staining.

tional signals (19). In the rat OX40L cDNA we cloned, three poly(A) additional signal sequence (AATAAA) were found at positions on 963, 1014, and 1772 (Fig. 1A). Therefore, it seems likely that the ~1.0 kb mRNA is generated by usage of the first or second poly(A) additional signal and the ~1.9 kb mRNA is generated by usage of the third poly(A) additional signal. The cDNA sequence we cloned (1804bp) appears to represent the ~1.9 kb mRNA.

Expression of OX40L on HTLV-1-Transformed Rat T Cell Lines

We next verified that the cDNA we cloned encodes the ligand for OX40 by estimating the binding of OX40-Ig fusion protein to the cDNA transfectants. The rat OX40L cDNA was subcloned into a mammalian expression vector and transfected into a normal rat kidney cell line NRK-52E that did not express OX40L mRNA (Fig. 2). The cDNA-transfected rOX40L/NRK cells, but not mock-transfected mock/NRK cells, bound OX40-Ig as estimated by flow cytometry (Fig. 3), indicating that the cloned cDNA does encode the ligand for OX40. As shown in Fig. 3, OX40-Ig also reacted strongly with HTLV-1-transformed T cell lines (F344-S1, ACI-S1, and WKA-S1), weakly with another HTLV-1-transformed T cell line (Lewis-S1), but not with a thymic lymphoma cell line (FTL13) or a macrophage cell line (WRT7/P2). The observed reactivity of OX40-Ig was in a good correlation with the levels of OX40L mRNA in these cell lines shown in Fig. 2. It has been reported that HTLV-1-transformed human T cell lines expressed OX40L constitutively, possibly due to the transactivation by Tax encoded by the *pX* region of HTLV-1 (6, 19, 21, 30). Tax has been shown to transactivates not only the viral long terminal repeat but also the expression of various host genes, including IL-2 receptor, IL-2, c-fos, c-jun, and OX40, and to play an essential role in HTLV-1-induced cellular transformation (20, 21). It seems likely that the expression of OX40L in the HTLV-1-transformed rat T cell lines also results from the transactivation by Tax.

Costimulatory Activity of Rat OX40L on T Cell Proliferation and Cytokine Production

It has been reported that mouse OX40L cDNA transfected CV-1/EBNA cells co-stimulated the proliferation of PHA-stimurated murine splenic T cells (5). Human OX40L cDNA transfected COS7 cells also co-stimulated the proliferation of human $CD4^+$ T cells stimulated by PMA, PHA, and anti-CD3 antibody (6). Then, we examined the co-stimulatory effect of rat OX40L on the proliferation and of anti-CD3-stimulated rat T cells. Rat splenic T cells were co-cultured with graded numbers of rOX40L/NRK or mock/NRK cells in presence of anti-CD3 mAb, and the proliferative responses were assessed after 3 days. As shown in Fig. 4A, the rOX40L/NRK cells co-stimulated the proliferation of anti-CD3-stimulated rat T cells in a dose-dependent manner. In contrast, no co-stimulatory effect was observed with the mock/NRK cells. We also measured IL-2 production in the culture supernatants by using the CTLL-2 proliferation assay. As shown in Fig 4B, the culture supernatant of rat T cells stimulated with anti-CD3 and rat OX40L resulted in enhanced proliferation of CTLL-2 cells, which was blocked by addition of anti-CD25 mAb. This indicated that rat OX40L co-stimulates IL-2 production by anti-

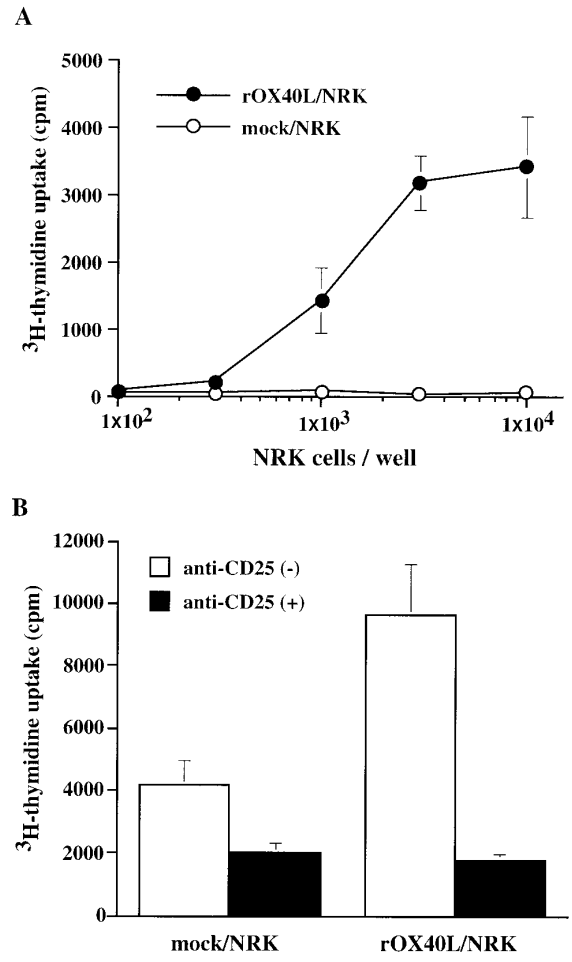

FIG. 4. Costimulatory effect of rat OX40L on rat T cell proliferation and IL-2 production. (A) SD rat splenic T cells (5×10^5/well) were cocultured with the indicated numbers of irrdiated rOX40L/NRK (●) or mock/NRK (○) cells in the presence of anti-CD3 mAb for 72 h. Proliferative response was assessed by pulsing the cultures with 0.5 μCi/well [^{3}H]thymidine for the last 6 h. (B) SD rat splenic T cells (5×10^5/well) were cocultured with irrdiated rOX40L/NRK or mock/NRK cells (1×10^4/well) in the presence of anti-CD3 mAb for 72 h. IL-2 production into the culture supernatants was determined by CTLL-2 proliferation assay. CTLL-2 cells (1×10^4) were incubated with the supernatants in the presence (■) or absence (□) of anti-CD25 mAb and 0.5 μCi/well of [^{3}H]thymidine was added 4 h before cell harvest. Data are expressed as mean ± SD of triplicate wells. Similar results were obtained from three independent experiments.

CD3-stimulated rat T cells. These results indicated that rat OX40L can provide an efficient co-stimulation for rat T cells, as observed in the mouse and human systems.

In the present study, we performed initial characterization of rat OX40L by molecular cloning. The structural features and co-stimulatory properties of rat OX40L were common to those of human or mouse counterparts. Notably, aberrant expression of rat OX40L was found in HTLV-1-transformed T cell lines as has been noted in the human system. It has been known that peripheral blood lymphocytes from HTLV-1-infected individuals exhibit spontaneous proliferation, which may be implicated in the development of adult T cell leukemia (ATL) (31,32). The co-stimulatory activity of OX40L expressed on the HTLV-1-infected T cells may contribute to such a process. HTLV-1 is not only an etiological agent of ATL but also causally to HTLV-1-associated diseases, such as myelopathy, uveitis, arthropathy, and Sjögrenis syndrome (33). A unique model of HTLV-1 infection has been developed in the rat system, which will be useful as an animal model for elucidating the involvement of OX40/OX40L in the pathogenesis of ATL and HTLV-1-associated diseases. OX40 has been also implicated in EAE (a model of multiple sclerosis) and RA (16). The present identification of rat OX40L enables us to define the pathophysiological roles of the OX40/OX40L system in rat models of autoimmune diseases and virus-associated diseases.

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