The Human OX40/gp34 System Directly Mediates Adhesion of Activated T Cells to Vascular Endothelial Cells

By Akihiro Imura, Toshiyuki Hori, Kazunori Imada, Takayuki Ishikawa, Yuetsu Tanaka,* Michiyuki Maeda,‡ Sadao Imamura,§ and Takashi Uchiyama

From Laboratory of AIDS Immunology, Research Center for Immunodeficiency Virus, The Institute for Virus Research, Kyoto University, Kyoto 606; the *Department of Biosciences, School of Science, Kitasato University, Kanagawa 228; ‡Chest Disease Research Institute, Kyoto University, Kyoto 606; and the §Department of Dermatology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

Summary

Fresh leukemic cells from patients with adult T cell leukemia (ATL) and some ATL-derived T cell lines show adhesion to human umbilical vein endothelial cells (HUVECs) mainly through E-selectin, but a proportion of this binding remains unaffected by the addition of combinations of antibodies against known adhesion molecules. By immunizing mice with one of such cell lines, we established monoclonal antibodies (mAbs), termed 131 and 315, that recognize a single cell surface antigen (Ag) and inhibit the remaining pathway of the adhesion. These mAbs did not react with normal resting peripheral blood mononuclear cells (PBMC) or most of the cell lines tested except for two other human T cell leukemia virus type I (HTLV-I)–infected T cell lines. After stimulation with phytohemagglutinin (PHA), PBMC expressed Ag 131/315 transiently, indicating that these mAbs define a T cell activation Ag. Western blotting and immunoprecipitation revealed that Ag 131/315 has an apparent molecular mass of 50 kD. Expression cloning was done by transient expression in COS-7 cells and immunological selection to isolate a cDNA clone encoding Ag 131/315. Sequence analysis of the cDNA indicated that it is identical to human OX40, a member of the tumor necrosis factor/nerve growth factor receptor family. We then found that gp34, the ligand of OX40, was expressed on HUVECs and other types of vascular endothelial cells. Furthermore, it was shown that the adhesion of CD4$^+$ cells of PHA-stimulated PBMC to unstimulated HUVECs was considerably inhibited by either 131 or 315. Finally, OX40 transfectants of Kit 225, a human interleukin 2–dependent T cell line, were bound specifically to gp34 transfectants of MMCE, a mouse epithelial cell line, and this binding was blocked by either 315 or 5A8, an anti-gp34 mAb. These results indicate that the OX40/gp34 system directly mediates adhesion of activated T cells or OX40$^+$-transformed T cells to vascular endothelial cells.

The trafficking and localization of circulating lymphocytes are regulated by interaction between several pairs of adhesion molecules expressed on lymphocytes and vascular endothelial cells. Tissue specificity of lymphocyte homing has been explained by selective expression of certain sets of adhesion molecules on subsets of lymphocytes as well as tissue-specific high endothelial venules. In mice, the molecules involved in each step of cell adhesion, namely, cell attachment, rolling, and adhesion (sticking), have been identified in different organs and tissues under ex vivo conditions (1–8). In humans, where umbilical vein endothelial cells (HUVECs)[1] are commonly used for in vitro experiments, at least three combinations of adhesion molecules are known to be important: LFA-1/intercellular cell adhesion molecules 1 and 2 (ICAM-1,-2); very late antigen 4 (VLA-4)/ vascular cell adhesion molecule 1 (VCAM-1), and several

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[1]Abbreviations used in this paper: ATL, adult T cell leukemia; HAEC, human aortic endothelial cell; HDMEC, human dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule 1; NGF, nerve growth factor; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

sialylated Ags such as cutaneous lymphocyte antigen (CLA)/ selectins (9–17). It is also indicated that CD6, CD44, and some other molecules can contribute to cell adhesion in certain circumstances (18–22).

Although not yet confirmed, there have been reports suggesting the presence of unknown adhesion pathways (23, 24). Our group previously reported that fresh leukemic cells from patients with adult T cell leukemia (ATL), which often manifests infiltration of malignant cells into a variety of organs such as skin, lung, and intestine, adhere to HUVECs predominantly through E-selectin and VCAM-1 expressed on endothelial cells (25–28). Data were presented implying that some part of this adhesion is likely to be mediated by unknown pathway(s) since it was only partially blocked by combinations of mAbs against LFA-1, VLA-4, E-selectin, and other known adhesion molecules. Further characterization was hindered because of limited availability and variation of fresh leukemic cell samples. However, we have recently found that among HTLV-I–infected human T cell lines, ATL-43T (Maeda, M., unpublished results), a human double negative T cell line derived from a patient with ATL, maintains high levels of unknown adhesion activity to HUVECs. It adheres considerably to unstimulated HUVECs, which cannot be blocked by combinations of mAbs against known adhesion molecules.

Recent evidence has disclosed that the phenomenon of cell adhesion is comprised of complicated sequential events involving ligand receptor recognition and subsequent cellular activation. Several cytokines, as well as membrane-bound costimulatory molecules, augment cell adhesion by upregulating the expression or bindability of certain adhesion molecules (29, 30). It is now widely accepted that not only cytokines but also adhesion molecules, upon binding to their ligands, give rise to activation signals that lead to the next step of cell adhesion (31–37). Furthermore, some cytokines of membrane-bound form have been shown to transmit signals into the cell just like cytokine receptors or costimulatory molecules (38, 39). These findings as a whole have brought about the situation where the distinction between the adhesion molecule and the costimulatory molecule or between the ligand and the receptor is not always self-evident. The next remaining question is whether the binding of any of the membrane-bound cytokines to their receptors or costimulatory molecules to their ligands directly mediates cell adhesion. Although there have been no definitive reports so far concerning this issue, we think that it is theoretically possible and deserves serious consideration.

In the present study, we developed mAbs against a cell surface Ag on ATL-43T that can inhibit its binding to HUVECs. We characterized these mAbs and cloned a cDNA encoding the antigen molecule recognized by these mAbs, which revealed that it is identical to the human counterpart of rat OX40, a type I transmembrane protein of the TNF/nerve growth factor (NGF) receptor family (40, 41). Recently, the ligand for OX40 has been identified as gp34, which was initially reported as an Ag expressed preferentially on HTLV-I–infected T cell lines (42–46). Here we present

evidence that the OX40/gp34 receptor ligand system, which has been described as a pair of TNF receptor/TNF family molecules with costimulatory function, directly mediates the binding of activated T cells to HUVECs.

Materials and Methods

Cells and Culture Conditions. IMDM medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (Summit Biotechnology, Ft. Collins, CO) and 30 μg/ml tobramycin (Shionogi Pharmaceutical Co., Osaka, Japan) was used throughout this study unless otherwise stated. ATL-43T is an IL-2–dependent HTLV-I–infected human T cell line with a phenotype of CD3$^+$, CD4$^-$, CD8$^-$ established from an ATL patient with intestinal lymphoid involvement. Kit 225 is an IL-2–dependent human T cell line established in our laboratory from a patient with T cell–type chronic lymphocytic leukemia (47). These cell lines were maintained in medium with 0.5 nM recombinant IL-2 (kindly provided by Shionogi Pharmaceutical Co). PBMC were isolated from heparinized venous blood from normal donors by Ficoll-Paque density gradient centrifugation. PBMC were used freshly or after culture with 0.1 μg/ml PHA (Murex Diagnostics Limited, Dartford, UK) for 3 d (PHA blasts). CD4$^+$ PHA blasts were prepared by negative selection using immunomagnetic beads (Dynabeads® M-450; Dynal, Oslo, Norway) after incubation with anti-CD8, anti-CD11b, anti-CD14, anti-CD16, and anti-CD20 mAbs. HUVECs were isolated by collagenase digestion according to the procedure of Jaffe et al. (48). In brief, umbilical cords obtained at normal deliveries were washed with 70% ethanol. The umbilical veins were rinsed with PBS and treated with 0.1% collagenase/PBS for 30 min at room temperature. Subsequently, the collagenase suspension was harvested and HUVECs were obtained by centrifugation. The pelleted cells were suspended in M199 medium (GIBCO BRL) containing 15% FCS and were incubated in a gelatin-coated flask (Corning Glass Works, Corning, NY). After incubation for 6 h at 37°C, the endothelial cells were washed and cultured in M199 medium supplemented with 15% FCS, 90 μg/ml porcine intestinal heparin (Sigma Chemical Co., St. Louis, MO), 30 μg/ml endothelial cell growth supplement (Collaborative Research, Inc., Bedford, MA), and antibiotics. HUVEC monolayers were then maintained in M199 medium containing the same additives in the second through the fourth passages and were used in the subsequent assays. The endothelial nature of the cells was confirmed by examining their morphological confluence and by detecting the expression of von Willebrand factor using an immunofluorescence assay. Weak expression of ICAM-1 and no expression of VCAM-1 and E-selectin on the resting HUVECs were demonstrated by flow cytometric analysis, whereas the induction of marked expression of these three molecules was noted after activation with 10 ng/ml of IL-1β (kindly provided by Otsuka Pharmaceutical Co., Tokyo, Japan). Human aortic endothelial cells (HAECs) and human dermal microvascular endothelial cells derived from the neonatal foreskin (HDMECs) were purchased from Kurabo (Osaka, Japan) and cultured as described above.

mAbs. mAbs were produced as described elsewhere (49). In brief, spleen cells from female BALB/c mice immunized with ATL-43T cells were fused with PAI mouse myeloma cells by polyethylene glycol (Sigma Chemical Co.) and subjected to selection in HAT medium (Sigma Chemical Co.). Hybridoma supernatants were screened based on difference in reactivity with original ATL-43T and one of its sublines that had lost adhesion activity to HUVECs after a long-term in vitro culture. The selected su-

pernatants were then tested for inhibitory effects on the binding of ATL-43T cells to HUVECs. Two independent mAbs, 131 and 315, were found to satisfy these criteria. mAbs 131 and 315 were typed as IgG1 and IgG2a, respectively, using a mouse Ig typing kit (Sigma Chemical Co.) and purified from ascitic fluids using a mouse IgG purification kit (Amersham, Tokyo, Japan). TS1/18 (anti-CD18, the β chain of LFA-1), W6/32 (anti-MHC class I, control IgG1), Lym-1 (anti-CD20), OKM-1 (anti-CD11b), and OKT8 (anti-CD8) were also purified from ascitic fluids of the hybridomas obtained from the American Type Culture Collection (Rockville, MD). HP2/1 (anti-CD49d, the α chain of VLA-4) was purchased from Immunotech S.A. (Marseille, France). 7A9 (anti-E-selectin) was kindly provided by Dr. W. Newman (Otsuka America Pharmaceutical Inc., Rockville, MD). 3G8 (anti-CD16) was a kind gift of Dr. J.C. Unkeless (Mount Sinai Medical Center, New York). TÜR14 (anti-CD14) was purchased from DAKO (Glostrup, Denmark). Anti-gp34 mAbs, 5A8, 8F4, TAG34, and TARM34 were described elsewhere (44–46).

Immunofluorescence Staining and Cytometric Analysis. Cells were stained by direct or indirect immunofluorescence and analyzed using a FACScan® (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (50).

Cell Adhesion Assay. HUVECs were plated onto gelatin-coated 96-well plates and cultured until confluence with or without prior exposure to 10 ng/ml of IL-1β (Otsuka Pharmaceutical Co.) for 4 h at 37°C. The cells were then rinsed twice with IMDM supplemented with 10% FCS. 10^5 ^{51}Cr-labeled T cells in a final volume of 60 μl were added to each well of HUVECs. After being left for 15 min at 37°C, the HUVECs were washed gently four times with prewarmed IMDM/10% FCS medium, and the remaining adherent cells were lysed with 2% NP-40 in PBS. One half of this volume was counted in a Top Count (Packard Instrument Co., Inc., Meriden, CT). Percent adhesion was calculated as the percentage of the count of remaining adherent cells to the count of input cells lysed with 2% NP-40 in PBS. To examine the effect of various mAbs on the binding of T cells to IL-1–stimulated or unstimulated HUVECs, ^{51}Cr-labeled T cells and HUVECs were incubated separately with supernatants of hybridomas or a saturating concentration (final concentration, 10 μg/ml) of each mAb for 10 min at room temperature after incubation with 5 μg/ml human IgGs to block the nonspecific binding to Fc receptors. T cells were then added to the wells of HUVECs. All experiments were performed in triplicate.

Western Blotting. Western blotting was done as described (49). Cell lysates from 5 × 10^6 cells were mixed with SDS sample buffer, boiled for 5 min, and electrophoresed through a 7.5–10% polyacrylamide gel. Samples were electrotransferred onto sheets of Immobilon-P (polyvinylidene fluoride) (Millipore Corp., Bedford, MA) membranes. The membranes were incubated serially with blocking buffer and with diluted mAb solutions, washed, and then incubated with rabbit peroxidase–conjugated anti–mouse IgG (American Qualex, La Mirada, CA). After washing, the binding of mAb was visualized by the enhanced chemiluminescence detection kit (ECL; Amersham).

Construction of cDNA Libraries. Poly(A)$^+$ RNA was prepared from 10^8 ATL-43T cells using a Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA). Oligo dT– and random hexamer–primed cDNAs were separately synthesized from 2 μg poly(A)$^+$ RNA and BstXI adaptors (Invitrogen) were added to each end. cDNA larger than 1 kb was selected by agarose gel electrophoresis and inserted into the BstXI sites of the expression vector pME18S (kindly provided by Dr. K. Maruyama, Tokyo Medical and Dental University, School of Medicine, Tokyo, Japan) (51). cDNA

was transformed into DH10B competent cells (GIBCO BRL) by electroporation. The oligo dT–primed and random hexamer–primed cDNA libraries consisted of 8 × 10^5 and 1.2 × 10^6 independent clones, respectively.

Expression Cloning. Expression cloning was done according to the method described by B. Seed and co-workers (52–54) except that plasmid DNA was introduced into COS-7 cells by electroporation (51). Oligo dT– and random hexamer–primed cDNA libraries were mixed 1:1 and 20 μg of plasmid DNA was transfected into 4 × 10^7 COS-7 cells. After 60 h of culture, cells expressing Ag 131/315 were immunologically selected using mAbs 131 and 315. Plasmid DNA was recovered as Hirt supernatants and transformed into DH5α competent cells by electroporation. After four cycles of transfection and immunologic enrichment, plasmid DNA was prepared from single colonies and transfected into COS-7 cells by the DEAE-dextran method. Transfected cells were examined for expression of Ag 131/315 by flow cytometry using a FACScan®.

Nucleotide Sequencing and Homology Search. The insert DNA was subcloned into pBluescript® II SK+ phagemid vector (Stratagene Inc., La Jolla, CA). Nucleotide sequences were obtained by the dyedeoxy termination method using Taq DyeDeoxy Terminator Cycle Sequencing on a model 373A automated sequencer (Applied Biosystems, Inc., Foster City, CA). Homology search was done using Genetyx Mac/CD ver. 29 (Software Development Co., Ltd., Tokyo, Japan).

Production of Stable Transfectants. cDNAs were subcloned into an expression vector pMKITneo (kindly provided by Dr. K. Maruyama) and transfected into Kit 225 cells or MMCE cells by electroporation. The expression vector for human gp34, pMKITneohgp34, was constructed as follows. The coding sequence of human gp34 was amplified by reverse transcription–PCR amplification of nucleotides 23-617 of human gp34 cDNA using total RNA extracted from MT-2 cells, and the primers 5'-CCCA-GATTGTGAAGATGGAA and 5'-GCCTGGTTTTAGATATT-GCC (55). The PCR product was inserted into pT7Blue vector (Invitrogen). This pT7hgp34 vector was digested with BamHI and XbaI, and 'ie resulting cDNA fragment was subcloned into the BamHI/XbaI site of pBluescript® SK+, generating pBluescript-hgp34. pMKITneohgp34 was constructed by transferring a XhoI/NotI fragment of pBluescript-hgp34 within pMKITneo vector in the sense orientation. Cells were centrifuged, resuspended in medium with 1 mg/ml Geneticin (GIBCO BRL), and dispensed 200 μl/well in 96-well plates. After 12–14 d, cultures were transferred to 24-well plates. Cells of each well were tested for expression of the relevant antigen by flow cytometry using a FACScan®.

Results

Generation of mAbs that Inhibit Binding of ATL-43T Cells to HUVECs. During the screening of HTLV-I–infected T cell lines for the unknown pathway(s) of adhesion, it was incidentally noticed that ATL-43T cells showed marked adhesion to HUVECs which was not inhibited by addition of any of the mAbs against LFA-1, VLA-4, or E-selectin. After a long-term in vitro culture, ATL-43T cells gradually lost binding activity to HUVECs in comparison with freshly thawed original ATL-43T cells. Therefore, we immunized BALB/c mice with original ATL-43T cells and screened hybridomas based on the quantitative difference
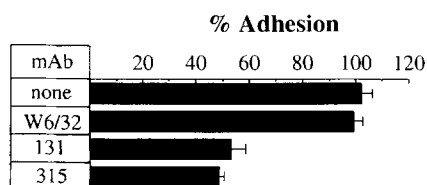
% Adhesion

mAb	20 40 60 80 100 120
none	
W6/32	
131	
315	

Figure 1. Effects of 131 as well as 315 hybridoma culture supernatants on binding of ATL-43T cells to HUVECs. The binding of ATL-43T cells to HUVECs was inhibited by hybridoma culture supernatants 131 as well as 315. ^{51}Cr-labeled ATL-43T cells were preincubated with medium alone, 10 μg/ml W6/32 mAb (anti-MHC class 1), or 1:1 dilutions of hybridoma culture supernatants for 10 min and then added to HUVECs precultured in 96-well flat-bottomed plates. The percentage of the remaining adherent cells to the total input cells was calculated as described in Materials and Methods.

in reactivity between original and long-term–cultured ATL-43T cells. Then we checked the effects of the supernatants of the selected hybridomas on binding of ATL-43T cells to HUVECs. mAbs 131 and 315 were found to exhibit significant inhibitory effects on cell adhesion between ATL-43T and HUVECs (Fig. 1). mAbs 131 and 315 were subtyped to be IgG1 and IgG2a, respectively. Immunoblotting and panel study of reactivity of these two mAbs clearly indicated that they recognize the same molecule as shown below.

Expression of Ag 131/315 on PBMC and Various Cell Lines. First we examined the expression of the Ags recognized by mAbs 131 and 315 on PBMC and various cell lines (Table 1). Neither 131 nor 315 reacted with normal fresh PBMC. However, as shown in Fig. 2, after stimulation with PHA, expression of Ag 131/315 was detected on day 1, reached maximal levels on days 2–3, and then declined. Two-color immunofluorescence analyses revealed that the major population expressing Ag 131/315 is the CD4$^+$ subset of PHA-stimulated PBMC, although some CD8$^+$ T cells weakly expressed this Ag (data not shown). Among the cell lines we tested, ATL-43T, Hut102, and MT-2, which are all HTLV-I–infected human T cell lines, were found to be positive for this Ag. Other T, B, myelomonocytoid, and (one) squamous cell lines were all negative. These data suggest that Ag 131/315 expression is restricted to activated T cells and may be induced in association with HTLV-I infection.

Biochemical Characterization of Ag 131/315. Western blotting was done to estimate the apparent molecular weight of the molecule recognized by mAbs 131 and 315. Consistent with the data of flow cytometry, a unique 50-kD band was detected in Hut102, ATL-43T, and PHA-stimulated PBMC by mAb 315 but not in Kit 225 or fresh PBMC (Fig. 3). Western blotting with mAb 131 gave a band of the same molecular weight (data not shown). Immunoprecipitation from day 3 PHA blasts by mAb 315 also showed a specific 50-kD band; after digestion with peptide N-glycosidase F (New England Biolabs, Beverly, MA), the immunoprecipitates migrated at the apparent molecular mass of ~40 kD, indicating that Ag 131/315 is a glycoprotein containing N-linked carbohydrate (data not shown).

Table 1. *Cell Surface Expression of 131/315 Ag*

Cell	Cell type	Distribution of 131Ag	315 Ag
ATL-43T	T	+*	+
Hut 102	T	+	+
MT-1	T	−	−
MT-2	T	+	+
Kit 225	T	−	−
MOLT-4	T	−	−
HPB-ALL	T	−	−
Jurkat	T	−	−
HSB-2	T	−	−
JY	B	−	−
Daudi	B	−	−
Raji	B	−	−
HeLa	Epithelial	−	−
HL60	Myeloid	−	−
U937	Monocytoid	−	−
HUVEC	Endothelial	−	−
Normal PBMC (n = 5)			
Fresh		−	−
PHA-stimulated (3 d)		+	+

*The cell surface expression of Ags 131 and 315 was examined by immunofluorescence staining and flow cytometry. The cutoff range was set in the fluorescence histogram of staining with control IgG at the right edge of the peak; percent positive cells was then determined with mAb 131 or 315. (−): positive cells <10%, (+): positive cells >10%.

Isolation and Identification of cDNA Encoding Ag 131/315. Since it seemed likely that mAbs 131 and 315 recognize an undefined adhesion molecule, we decided to isolate a cDNA clone encoding the Ag molecule by the expression cloning in COS-7 cells. After four cycles of transfection and immunologic enrichment, individual plasmid clones were transfected by the DEAE-dextran method into COS-7 cells, and after 48 h of culture, the expression of Ag 131/315 was examined by flow cytometry. One of the clones, pME18S315-02, containing a 1.1-kb insert, was found to encode the antigenic epitope recognized by both mAbs 131 and 315 (Fig. 4). The nucleotide sequence of the insert DNA was determined using an automated sequencer and the sequence was subjected to the homology search. To our surprise, it was identical to the cDNA of the human counterpart of rat OX40 (data not shown). The cDNA we cloned included the sequence from the 5′ end to a halfway point before the poly(A) tail of the published human OX40 cDNA. It is likely to encode the integral peptide as reported, which is supported by the fact that Western blotting of stable transfectants of our cDNA gave a band with the same molecular weight as PHA-stimulated normal PBMC (data not shown).
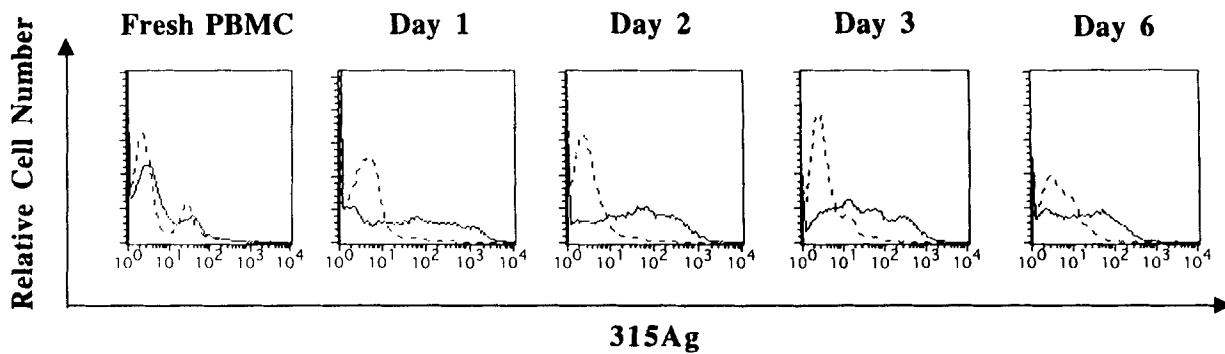
Figure 2. Expression of Ag 315 on PBMC after stimulation with PHA. Normal PBMC were cultured with 0.1 μg/ml PHA, and the expression of Ag 315 (*solid line*) at the indicated time points was measured by indirect immunofluorescence followed by flow cytometric analysis. Control indicates staining with irrelevant mouse IgG2a antibody (*dotted line*).

Anti-OX40 mAbs Inhibit Binding of CD4⁺-activated T Cells to Vascular Endothelial Cells. As mentioned before, OX40 was identified as an activation Ag predominantly expressed on the CD4$^+$ population of activated T cells (40). To clarify whether OX40 can function as a cell adhesion molecule for activated T cells, we performed the inhibition study with mAb 315 using CD4$^+$ cells purified from PHA-stimulated PBMC. We first confirmed that the CD4$^+$ PHA blasts expressed high levels of OX40 in flow cytometric analysis (data not shown). The cells were then analyzed for adhesion activity to both resting and IL-1–stimulated HUVECs in the presence or absence of mAbs. Three independent experiments were done with samples from three different healthy volunteers, and a representative experiment is shown in Fig. 5. A considerable proportion of the cells were bound to unstimulated HUVECs. It was noted that 315 (anti-OX40 mAb) rather than TS1/18 (anti-LFA-1 mAb) inhibited this adhesion and the addition of both 315 and TS1/18 blocked most of the binding. When IL-1–stimulated HUVECs were used, 315, TS1/18, HP2/1 (anti-VLA-4 mAb), and 7A9 (anti-E-selectin mAb) each showed significant but modest inhibition by itself. Combination of these mAbs clearly increased inhibitory effects on adhesion, and yet the effect of mAb 315 was prominent when added together with the other mAbs. Therefore, the cell adhesion to IL-1–stimulated HUVECs seems to be mediated by the several pairs of adhesion molecules and a significant part of this adhesion is ascribed to OX40-mediated binding. These data indicated that OX40 is involved in binding of activated CD4$^+$ T cells to HUVECs, whether or not HUVECs were stimulated. As to other types of vascular endothelial cells, binding of CD4$^+$ PHA blasts to HAECs or HDMECs and its inhibition by mAb 315 was similarly observed (data not shown).

The Ligand for OX40 on Vascular Endothelial Cells is gp34. Recent studies have demonstrated that the ligand of OX40 is gp34, a type II transmembrane protein of the TNF family that is expressed on some HTLV-I–infected T and activated B cells (42, 43). Therefore, we next examined if gp34 was expressed on HUVECs and other types of vascular endothelial cells. HUVECs, HAECs, and HDMECs were stained with anti-gp34 mAb and analyzed by flow cytometry. As

shown in Fig. 6, not only HUVECs but also HAECs and HDMECs expressed considerable levels of gp34 without any stimulation. In fact, no induction or augmentation of gp34 expression was detected after culture of these endothelial cells with IL-1β (data not shown). Western blotting was done to characterize the molecules expressed on HUVECs that were recognized by 8F4, an anti-gp34 mAb. A specific 34-kD band was seen by 8F4 in HUVECs and MT-2, indicating that HUVECs express the gp34 molecule of the same molecular weight as originally described in HTLV-I–infected T cells (Fig. 7). We then examined if anti-gp34 Ab could inhibit cell adhesion. As had been expected, 5A8, an anti-gp34 mAb, inhibited binding of ATL-
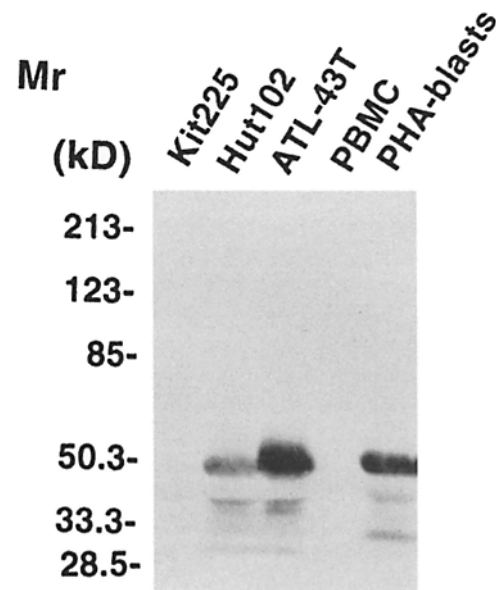

Figure 3. Western blotting of Ag 315. Cell lysates of Kit 225, Hut102, ATL-43T, fresh PBMC, and PHA-stimulated PBMC (day 3) were mixed with SDS-sample buffer, boiled for 5 min, and then applied to electrophoresis through a 7.5% polyacrylamide gel. Proteins were transferred to a sheet of PVDF membrane and the membrane was incubated with 10 μg/ml mAb 315. The binding of the mAb was detected as described in Materials and Methods.

Control   H107   131   315
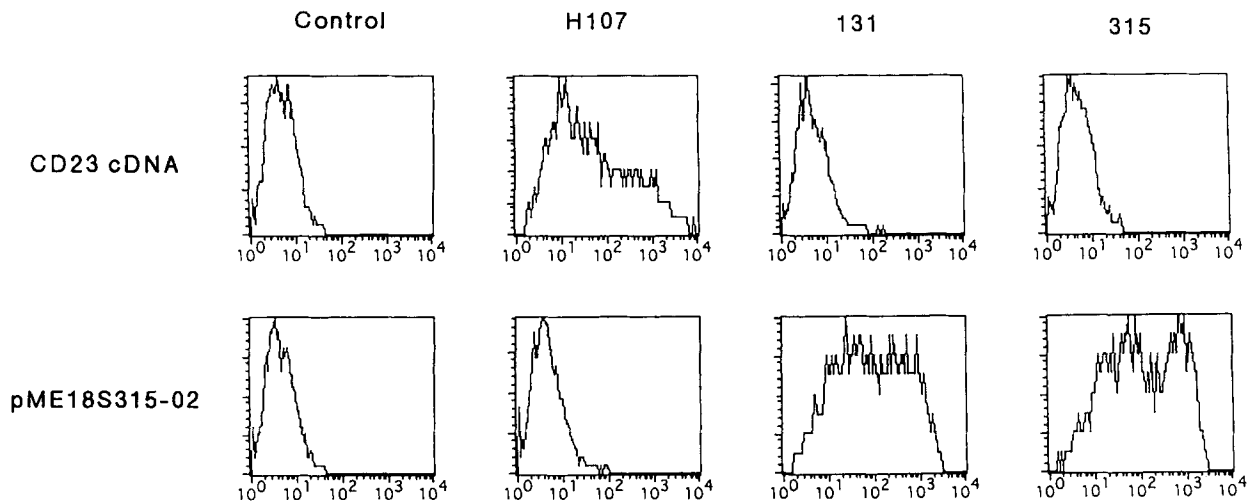

CD23 cDNA

pME18S315-02

Figure 4. Expression of Ags 131 and 315 on COS-7 cells transfected with the isolated cDNA clone. The isolated cDNA, pME18S315-02, as well as CD23 cDNA (*Control*) was transfected into COS-7 cells by the DEAE-dextran method. After 2 d of culture, cells were detached by 0.5 mM EDTA, stained with 10 μg/ml H107 (anti-CD23 mAb), 131, or 315 by indirect immunofluorescence, and subjected to flow cytometric analysis. Control indicates staining the second antibody alone.

43T to HUVECs in a dose-dependent manner (Fig. 8). The inhibition by mAb 5A8 was even slightly more efficient than that by mAb 315. These data clearly indicate that gp34 is the major OX40 ligand expressed on HUVECs.

The OX40/gp34 System Directly Mediates Cell Adhesion. Since both OX40 and gp34 have been reported to transmit stimulatory signals to the cell (42, 43), it is important to determine if the OX40/gp34 system mediates cell adhesion directly or indirectly by activating other adhesion molecules. To address this question, we made a stable OX40 transfectant of Kit 225 and a stable gp34 transfectant of MMCE, a mouse epithelial cell line (Fig. 9). Kit 225 exhibits almost no adhesion to MMCE presumably due to the difference in species. As shown in Fig. 10, OX40-transfected Kit 225 cells that did not bind to parental MMCE cells showed marked adhesion to gp34-transfected MMCE

cells, whereas no binding between parental Kit 225 cells and the gp34 transfectants were observed. To further confirm the role of the OX40/gp34 system in cell adhesion, a cell adhesion assay was done using paraformaldehyde-fixed cells (Fig. 11). Although fixation of either ATL-43T or HUVECs decreased the binding capacity, there remained some specific binding that was blocked by either anti-OX40 or anti-gp34 mAb. These results strongly indicate that the OX40/gp34 system directly mediates binding between OX40+ T cells and gp34+ vascular endothelial cells.

Discussion

OX40 was first identified on activated rat CD4+ T cells (40). During the past several years, cDNAs encoding rat, mouse, and human OX40 have been cloned (41, 56, 57). It
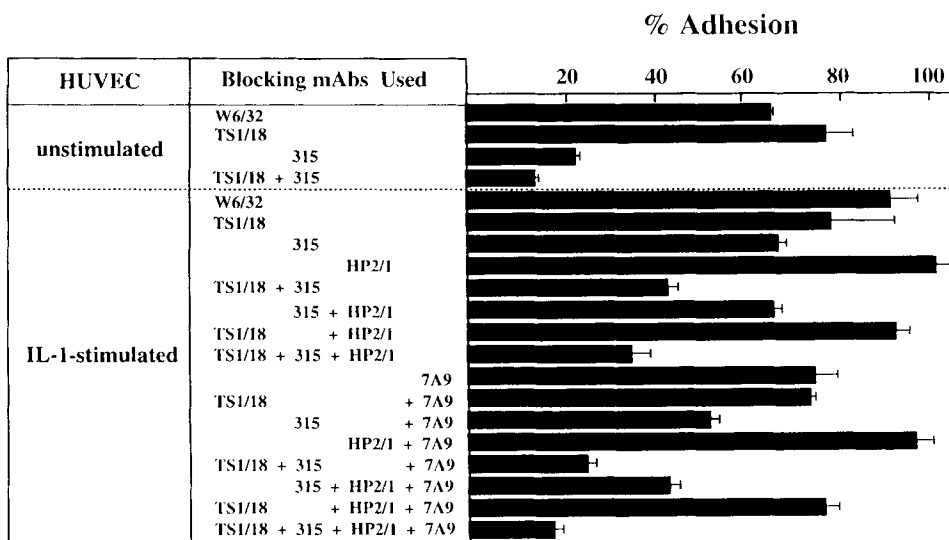

Figure 5. Effects of anti-LFA-1, anti-VLA-4, anti-E-selectin, and anti-OX40 mAbs on the binding of CD4+ PHA-stimulated PBMC to HUVECs. Purified CD4+ cells from PHA-stimulated PBMC (day 3) were preincubated with 10 μg/ml of W6/32 (anti-MHC class I mAb), TS1/18 (anti-CD18 mAb), 315 (anti-OX40 mAb), or HP2/1 (anti-VLA-4 mAb). HUVECs were also preincubated with 10 μg/ml of 7A9 (anti-E-selectin mAb). The binding to unstimulated or IL-1-stimulated HUVECs was then determined as described in Materials and Methods.
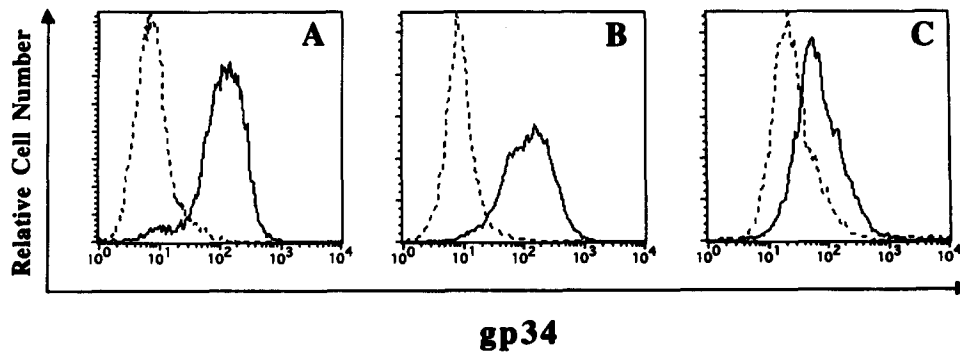
Figure 6. Expression of gp34 on vascular endothelial cells. HUVECs (*A*), HAECs (*B*), and HDMECs (*C*) cultured in vitro were stained with 5A8 (anti-*gp34* mAb) by indirect immunofluorescence and subjected to flow cytometric analysis. (*Dotted lines*) Staining with control mouse IgG2a antibody.

has been shown that OX40 is a member of the TNF/NGF receptor family, which also includes CD40, Fas (APO-1), CD30, CD27, and 4-1BB/ILA (58–60). The members of this family are type I membrane molecules and contain in their extracellular domain three to four imperfect repeats of ~40 residues, anchored by a superimposable pattern of six cysteines. Expression of OX40 is restricted to activated T

cells, and the binding of OX40 to its ligand, gp34, has been reported to result in increased T cell proliferation in the presence of suboptimal doses of mitogens (42, 43). Thus, OX40 has been assumed to be a kind of cytokine receptor with costimulatory function whose ligand is membrane bound (42, 43, 61).

In the present study, we developed mAbs 131 and 315 that inhibit adhesion between ATL-43T, an ATL-derived T cell line, and HUVECs. Characterization of the Ag molecule and subsequent cDNA cloning revealed that the 50-kD glycoprotein recognized by these mAbs is identical to human OX40. It was unexpected that OX40, a member of the NGF/TNF receptor family, is involved in binding of ATL-43T cells to HUVECs. We have shown that the binding of CD4$^+$ PHA-stimulated PBMC to HUVECs is also inhibited by anti-OX40 mAbs. In particular, their binding to unstimulated HUVECs is practically blocked by one of the anti-OX40 mAbs alone.

gp34, the ligand of OX40, was previously described as a surface molecule expressed on certain HTLV-I–infected T cell lines (44–46). Although it is known that gp34 is expressed in multiple organs such as heart, skeletal muscle, testis, and activated B cells (42), expression of gp34 in vari-
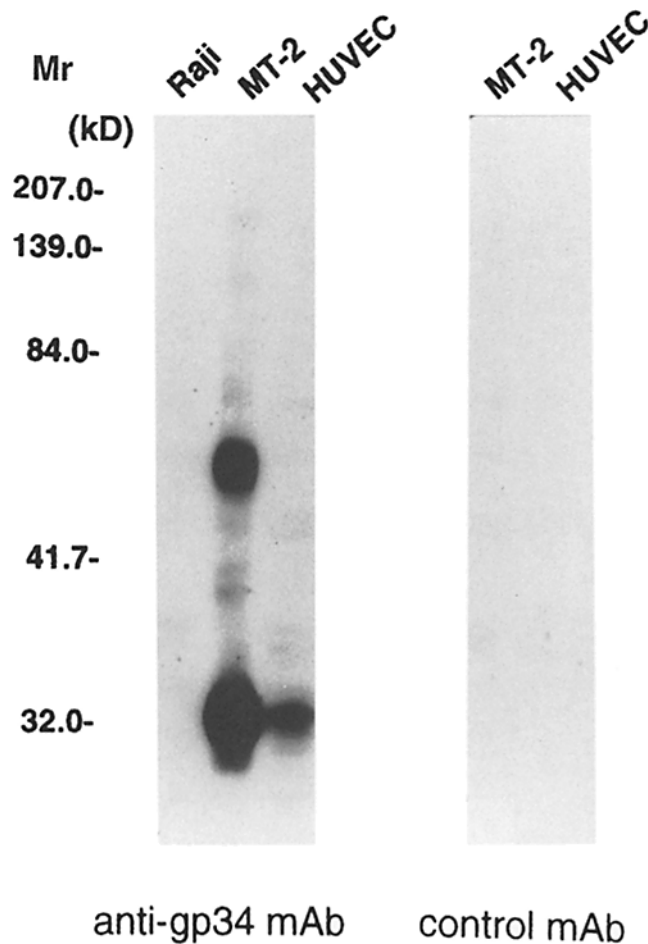

Figure 7. Western blotting of gp34 expressed in HUVECs. Cell lysates of HUVECs, MT-2 cells, or Raji cells were subjected to SDS-PAGE and transferred to a sheet of PVDF membrane. The membrane was incubated with 10 μg/ml 8F4 (*anti-gp34 mAb*) or negative control mAb. The binding of mAb was detected as described in Materials and Methods.
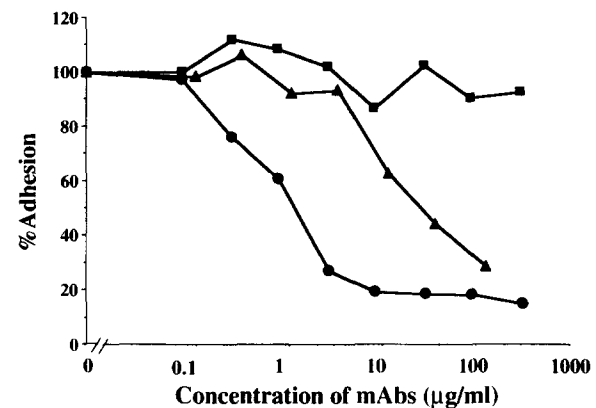

Figure 8. Effects of anti-OX40 as well as anti-gp34 mAb on binding of ATL-43T cells to HUVECs. ATL-43T cells were preincubated with serial dilutions of W6/32 (anti-MHC class I ■), 315 (anti-OX40 mAb ▲), or 5A8 (anti-gp34 mAb ●) and then subjected to the adhesion assay. % Adhesion was formulated as a percentage of the binding with mAb to that without mAb.
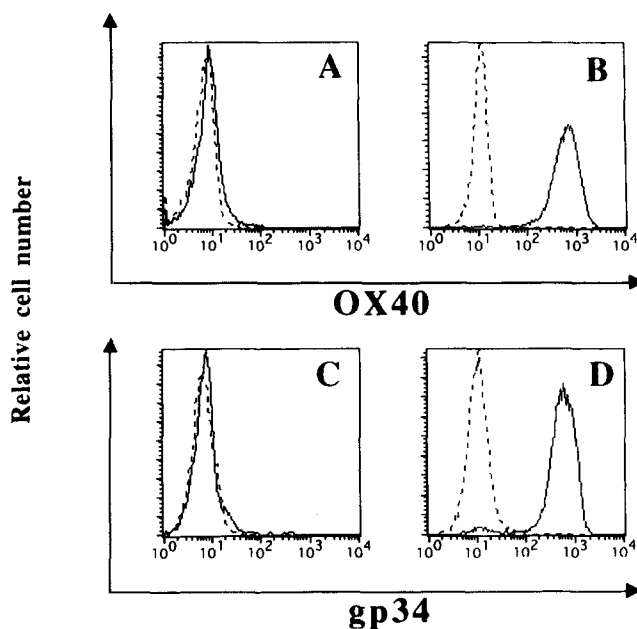
Figure 9. Expression of OX40 and gp34 on the stable transfectants. Kit 225 (*A*), a subline of Kit 225 stably transfected with pMKITneo containing OX40 cDNA (*B*), MMCE (*C*), and MMCE stably transfected with pMKITneo containing gp34 cDNA (*D*) were stained with 315 (anti-*OX40* mAb) or 5A8 (anti-*gp34* mAb) by indirect immunofluorescence and subjected to flow cytometric analysis. (*Dotted lines*) Staining with control mouse IgG2a antibody.

ous tissues and cell types has not been investigated in detail. Here we report for the first time that human vascular endothelial cells including HUVECs, HAECs, and HDMECs, express gp34 without stimulation in vitro. Furthermore, we showed that not only anti-OX40 mAb but also anti-gp34 mAb inhibited binding of ATL-43T to HUVECs, indicating that the OX40/gp34 system participates in cell adhesion.

As to the mechanism of cell adhesion mediated by the OX40/gp34 system, the following possibility should be taken into consideration. OX40/gp34 interaction may generate stimulatory signals resulting in increased binding ac-

tivity of the other existing adhesion molecules. Indeed, Hollenbaugh et al. (62) have reported that the interaction of CD40 expressed on vascular endothelial cells with its ligand can lead to endothelial cell activation, which in turn leads to leukocyte adhesion through the upregulation of ICAM-1, VCAM-1, or E-selectin. A similar explanation also seems possible with the OX40/gp34 system. Nevertheless, it is rather unlikely that the major part of cell adhesion shown in this study is indirectly mediated by stimulatory signals through the OX40/gp34 system. First, incubation time in adhesion assay is too short to cause a quantitative change in expression of other adhesion molecules. Second, cell adhesion mediated by OX40 and gp34 could be reconstructed in OX40-transfected Kit 225 and gp34-transfected MMCE, a mouse epithelial cell line lacking human ICAM-1, VCAM-1, and E-selectin. This adhesion was specifically inhibited by anti-OX40 or anti-gp34 mAb. Finally, paraformaldehyde-fixed ATL-43T cells still showed modest but significant binding to nonfixed or fixed HUVECs, which was inhibited by anti-OX40 as well as anti-gp34 mAb.

It is now realized that cell adhesion is a multistep process involving ligand receptor recognition and subsequent cellular activation. Most of the adhesion molecules themselves have recently been shown to transmit activation signals (29–31). A number of cytokines and costimulatory molecules are also involved in this process by upregulating expression or bindability of adhesion molecules. However, there is another aspect of the ligand receptor interaction: the intercellular binding between membrane molecules is always accompanied by physical cell-to-cell binding. Accordingly, it is rather natural to presume that binding of a membrane-bound molecule to its ligand or receptor contributes to cell-to-cell binding to some extent, whether or not this contribution can be detected, depending on the assay system and coexistence of other adhesion molecules. Our study presents such an interesting case in which a cytokine receptor–like molecule, OX40, and its ligand, gp34, can directly mediate cell adhesion. Remodeling of the concepts of adhesion and costimulatory molecules will be required to reconcile these results.

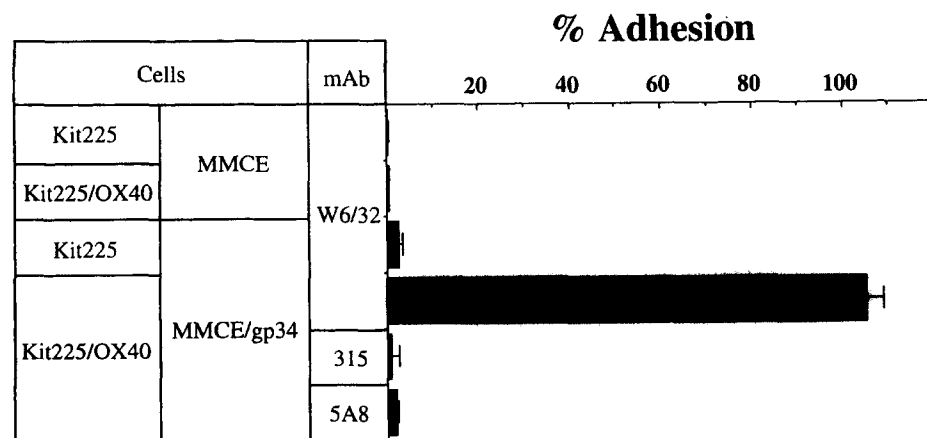T cell recruitment has been investigated based on the

% Adhesion

Cells		mAb	20	40	60	80	100
Kit225	MMCE	W6/32					
Kit225/OX40							
Kit225	MMCE/gp34						
Kit225/OX40							
		315					
		5A8					

Figure 10. Reconstitution of the binding mediated by OX40 and gp34 in the stable transfectants. Binding of Kit 225 or its stable transfectants of OX40 (*Kit225/OX40*) to MMCE or its stable transfectants of gp34 (*MMCE/gp34*) was measured in the presence or absence of mAbs. Kit 225 or Kit 225/OX40 cells were preincubated with medium alone, W6/32 (anti-MHC class I mAb), 315 (anti-OX40 mAb), or 5A8 (anti-gp34 mAb), and subjected to the adhesion assay.

%Adhesion

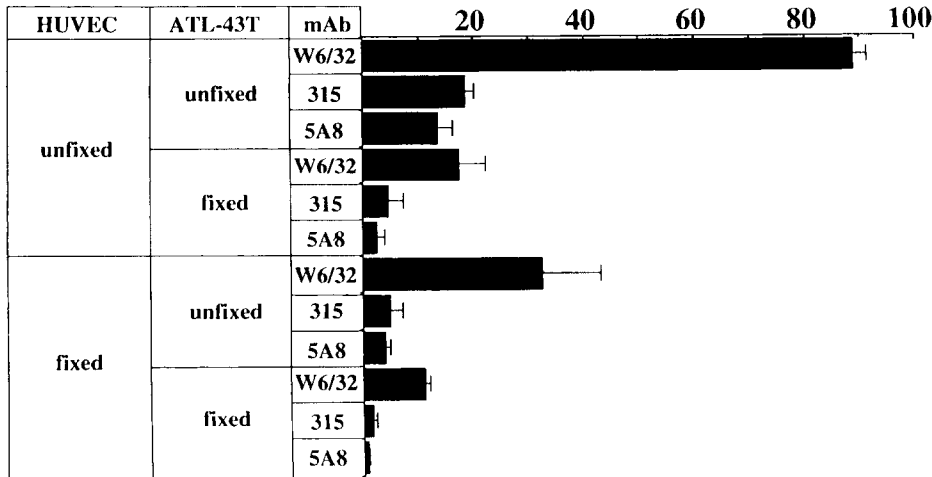| HUVEC	ATL-43T	mAb
unfixed	unfixed	W6/32
		315
		5A8
	fixed	W6/32
		315
		5A8
fixed	unfixed	W6/32
		315
		5A8
	fixed	W6/32
		315
		5A8

Figure 11. Adhesion between ATL-43T and HUVECs after fixation with paraformaldehyde. ATL-43T cells, unfixed or fixed with 0.4% paraformaldehyde for 10 min at room temperature, were preincubated with 10 µg/ml of W6/32 (anti-MHC class I mAb), 315 (anti-OX40 mAb), or 5A8 (anti-gp34 mAb) and then their binding to HUVECs, unfixed or fixed with 0.4% paraformaldehyde, was measured.

hypothesis that several pairs of homing receptors and vascular addressins mediate adhesion of lymphoid cells to endothelial cells and regulate their localization. Therefore, tissue specificity of the homing of T cell subsets is thought to be determined predominantly by cell type— or tissue-specific expression of a certain set of adhesion molecules including integrins, Ig superfamily, and selectins. The OX40/gp34 system seems to define a distinct pathway of T cell recruitment that is independent of the tissue specificity or the conditions of tissue environment, affecting any type of activated CD4$^+$ T cells. It may be complementary to the conventional tissue-specific cell adhesion, especially in situations where a significant proportion of circulating T cells are activated and express OX40.

Finally, the present study reemphasizes the importance of vascular endothelial cells for distribution and activation of T cells. It is very likely that interaction between T cells and vascular endothelial cells via OX40 and gp34 generates activation signals to both sides in addition to the physical cell adhesion. Further studies are required to delineate its physiologic as well as pathologic meanings and the roles of vascular endothelial cells during this interaction for the better understanding of immune response in vivo.

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