Characterization of adhesion receptors on cultured microvascular endothelial cells derived from the retroorbital connective tissue of patients with Graves' ophthalmopathy

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T lymphocytes have been demonstrated recently to play an important role in the pathogenesis and propagation of Graves' ophthalmopathy (GO). Recruitment of T cells to the retroorbital tissue in GO involves the activation of certain adhesion molecules both in the vascular endothelium and in the extravascular connective tissue within the retroorbital space. To characterize the interactions between orbital endothelial cells (OECs) and circulating T cells in vitro, we designed a two-step immunopurification procedure with bead-immobilized Ulex europaeus I lectin and anti-human endothelial cell antigen (CD31) monoclonal antibody for rapid and reproducible isolation of highly pure microvascular endothelial cell populations from small quantities of retroorbital connective tissue. Endothelial origin of the resulting cell populations was confirmed by positive immunoreactivity for von Willebrand factor, CD31 and thrombomodulin. Under baseline conditions, GO-OECs, but not normal OECs, expressed intercellular adhesion molecule 1 (ICAM-1) and CD44 immunoreactivity but no immunoreactivity for endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was detected. Exposure of GO-OEC and normal OEC monolayers to interferon γ, interleukin 1α and tumor necrosis factor α resulted in marked up-regulation of immunoreactivity for ICAM-1 and in induction of ELAM-1 and VCAM-1. Blocking experiments using monoclonal antibodies directed against various adhesion molecules demonstrated that interactions between matched activated T lymphocytes and OECs were mediated by integrin-dependent (ICAM-1 leukocyte function-associated antigen 1 (LFA-1): VCAM-1/early late antigen 4 (VLA-4)) and integrin-independent (CD44) pathways, and revealed marked differences when comparing GO-OECs and normal OECs. In conclusion, the availability of OECs from affected retroorbital tissue of patients with GO provides a valuable tool for studying further the mechanisms responsible for orbit-specific lymphocyte recruitment in GO.

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Graves' ophthalmopathy (GO), an inflammatory condition affecting the retroorbital space, represents the most common extrathyroidal manifestation of Graves' disease (1, 2). The histological hallmarks of GO include infiltration of the orbital tissues by inflammatory cells, proliferation of the orbital and perimysial connective tissue and the accumulation of hydrophilic glycosaminoglycans (GAGs) (3–5). These features are responsible for the prominent alterations of the retroorbital tissue compartment and play a central role in the evolution of GO, leading to the clinical manifestations of proptosis, diplopia, periorbital swelling and inflammation (2). Various cytokines, produced by infiltrating monocytes/macrophages, mast cells and lymphocytes, as well as resident connective tissue cells, are considered to act as local modulators of cellular immune and metabolic activities within the affected retroocular tissues (6). Cytokine effects of potential relevance to the orbital disease process in GO include, among others, the induction of MHC class II molecules, heat shock proteins and adhesion molecules in retroorbital fibroblasts (2, 3, 7–9). These cells are likely to be both target cells and effector cells in the ongoing immune process in GO (1, 10–12). Recent analyses of the retroorbital mononuclear cell infiltrates suggest that lymphocytes play an important role in the pathogenesis and propagation of GO (12, 13). In support of this concept, lymphocytes derived from the retroorbital tissue of patients with GO have been found to recognize specifically autologous retroorbital and, to a lesser degree, autologous skin fibroblasts, suggesting that retroorbital fibroblasts represent a major orbital target for T cells (12).

In order for T lymphocytes to target and activate retroorbital fibroblasts in the extravascular space, they have to migrate through the endothelial cell layer of the
retroorbital microvasculature prior to establishing cell contact with retroorbital fibroblasts. Thus, activation of certain adhesion molecules in vascular endothelial cells and in the surrounding extravascular tissue most likely plays an important role in the recruitment of immune-competent cells to the site of an autoimmune attack (14). However, the mechanisms of lymphocyte recruitment to the retroorbital space in GO have not been characterized in detail. In the absence of a valid animal model of GO, systematic investigation of the cellular mechanisms involved in these interactions requires carefully controlled in vitro experiments and availability of purified populations of the various cell types involved in situ.

To analyze the mechanisms of T-cell recruitment to the extravascular compartment of the retroorbital space, we have devised a technique that permits rapid and reproducible isolation of a highly homogenous population of microvascular endothelial cells from small quantities of retroorbital connective tissue. Orbital endothelial cells (OECs) isolated by this technique from patients with GO and normal individuals were characterized phenotypically and compared for the constitutive and inducible expression of adhesion molecules. Further, we have begun to characterize receptor/ligand interactions relevant for T-cell adhesion to microvascular endothelial cells within the retroorbital connective tissue of patients with GO.

Methods

Reagents

Recombinant cytokines were purchased from Genzyme Corp. (Boston, MA) and Boehringer Mannheim (Mannheim, Germany). Anti-intercellular adhesion molecule 1 (ICAM-1) monoclonal antibody was a generous gift from Dr Timothy Springer, Boston. Monoclonal antibodies directed against ICAM-1, endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1) were purchased from British Biotechnology Limited (Abingdon, UK). Monoclonal antibodies directed against vimentin, desmin, fibroblast antigen, thrombomodulin and CD31 were from Dakopatts Corporation (Santa Barbara, CA). A non-blocking monoclonal antibody specific for CD44 (CD44(A)) and a blocking monoclonal antibody directed against the hyaluronic acid binding site of CD44 (CD44(B)) were obtained from Dianova (Hamburg, Germany). Monoclonal antibodies against myosin and von Willebrand antigen, goat-anti-human IgG and 12-0-tetradecanoyl-phorbo1 13-acetate (TPA) were purchased from Sigma (St. Louis, MO). Ulex europaeus I lectin, monoclonal antibody against Ulex europaeus I lectin, biotinylated anti-mouse IgG antibody and the Vectastain ABC Elite detection system were obtained from Vector Laboratories (Burlingame, CA). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT) and Na$^{31}$CrO$_4$ was purchased from New England Nuclear (Boston, MA). All other reagents used were of the highest purity available commercially.

Isolation and culture of endothelial cells

 Orbital connective tissue specimens were obtained as surgical waste from patients during transantral orbital decompression (TAOD) for severe, inflammatory GO (N = 6). All patients had had hyperthyroid Graves’ disease and had been rendered euthyroid prior to TAOD. Patients had received glucocorticoids (60–80 mg/day, followed by tapering of doses) in the distant past, but not within 3 months prior to TAOD. In addition, orbital connective tissue specimens were obtained from five patients undergoing orbital surgery for unrelated conditions (strabismus surgery, refractory secondary glaucoma). Freshly obtained tissue specimens were transferred to the laboratory on ice, mechanically disaggregated and minced. The resulting tissue fragments were digested with pronase and resuspended in Hank’s balanced salt solution (HBSS) containing 15% FBS. Isolation of human OECs was performed using immunomagnetic polystyrene beads in a positive, direct selection procedure involving two successive rounds of immunopurification. Tissue homogenates were first incubated with 20μl of immunomagnetic particles covalently coated with Ulex europaeus I lectin by continuous gentle mixing for 20 min at 4°C. Immuno-bead/target complexes were immobilized to the side of the tube by application of a magnetic field using a magnetic particle concentrator. Following a series of five washes, rosetted bead-cell pellets were resuspended in HBSS/FBS + 0.01 mol/l fucose for 10 min at room temperature for detachment of beads from isolated cells. This cell population was collected by centrifugation and subjected to a second round of immunopurification, using a monoclonal antibody directed against a human endothelial cell-specific surface marker (CD31). The resulting OEC population was resuspended and propagated in endothelial cell growth medium (EGM: PromoCell, Heidelberg, Germany) containing 5% dialyzed FBS and antibiotics (gentamycin/amphotericin B) in a humidified incubator at 37°C with an atmosphere of 5% CO$_2$/95% air. All cell strains were used between the second and third cell passages. Prior to study, OEC monolayers were trypsinized, counted and seeded in culture dishes or multichamber slides.

Immunocytochemistry

Immunocytochemistry was performed using a highly sensitive three-stage immunoperoxidase protocol, as described previously (6, 7). In brief, equal cell numbers of OECs were plated directly onto 8 x 8 mm multichamber slides (Nunc, Naperville, IL).
resulting in cell monolayers of equal densities (approximately 80% confluency) following adherence of the cells. Endogenous peroxidase activity was inhibited and non-specific binding was blocked for 30 min with normal serum diluted 1:20 in phosphate-buffered saline (PBS) Phenotypical characterization of cells was performed using a panel of monoclonal antibodies directed against vascular endothelial cell surface markers, including ICAM-1, ELAM-1, VCAM-1, CD44s, vimentin, desmin, myosin, fibroblast antigen, thrombomodulin, CD31 and non Willebrand antigen. Primary antibodies were applied for 30 min, diluted in PBS (pH 7.4) containing 1% normal horse serum, at predetermined optimal concentrations. Biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) was applied for 30 min at 1:250 dilution in PBS containing 1% normal horse serum. Sections were then incubated for 30 min with avidin and biotinylated peroxidase according to the manufacturer's instructions (Vectastain Peroxidase ABC elite kit, Vector Laboratories) and immunoreactivity was detected using a standard peroxidase substrate system containing diaminobenzidine (DAB) as the chromogen. Parallel sections with the primary antibody replaced by unrelated antibodies, by non-immune mouse IgG of the same isotype and with the omission of either primary or secondary antibodies were examined to assure specificity and to exclude cross-reactivities between the antibodies and conjugates employed.

The percentage of OECs displaying positive immunoreactivity was evaluated by visual microscopy and differential cell counting of at least 250 individual cells in 10 randomly selected visual fields, according to Todd et al. (15). The examiner was unaware of the treatment and source of the cell monolayers.

Isolation and labelling of T cells

Matched peripheral blood lymphocytes were prepared from heparinized blood of patients and control individuals using Ficoll-Paque (Pharmacia) density gradient centrifugation, and T cells were purified by rosetting with neuraminidase-treated sheep erythrocytes. Cells were resuspended to 2 × 10^7 cells/ml in RPMI 1640 medium containing 5% FBS. The resulting T cell suspensions were > 95% CD3+ by flow cytometry and revealed > 95% viability by trypan blue exclusion. For visual counting of cell adherence, T cells were used either directly (unstimulated) or following stimulation with TPA at a concentration of 50 μmol/l for 30 min. For quantitation of cell attachment using radioactivity measurements, freshly prepared, unstimulated or TPA-treated T cells were labelled with 51Cr (0.3 mCi) for 2 h at 37°C, washed three times and adjusted to a cell density of 10^5 per 100 μl of medium prior to use in the adhesion assay.

Adhesion assay

Orbital endothelial cell–lymphocyte interactions were studied using a standard adhesion assay in which cytokine-activated OECs were co-cultured with matched T cells, as described previously (9). In brief, OECs were plated at approximately 5 × 10^4 cells per well on flat-bottom 24-well plates and grown to confluence. Monolayers were switched to EGM containing 1% FBS for 24 h prior to the adhesion studies and were > 80% confluent on the day of use. To compensate for possible variation in cell density, quadruplicate wells were evaluated in each experiment. Cytokines were added to the culture medium containing 0.1% PBS to the following concentrations: interleukin 1α (IL-1α, 10 U/ml), interferon γ (IFN-γ 100 U/ml), tumor necrosis factor α (TNF-α 100 U/ml), interleukin 2 (IL-2, 10 U/ml), transforming growth factor β (TGF-β 10 ng/ml). In previous experiments, designed to determine the dose dependency of retroorbital fibroblasts and OECs to cytokines, maximal stimulation of cellular functions, including cell proliferation and ICAM-1 expression, has been found to occur at these concentrations (9, 16). Monolayers were rinsed extensively with EGM prior to addition of T cells. Untreated and stimulated, labeled or unlabeled T cells were added to each well at a density of 10^5 cells per well and incubated in the presence of 1% human AB serum for 1 h at 37°C. Non-adherent lymphocytes were removed by gentle washing six times with EGM at 37°C, and the wells were aspirated to dryness without disturbing the monolayer. For adhesion analysis using optical microscopy, cells were fixed in 100% cold methanol for 15 min prior to cell counting. In the case of 51Cr-labeled peripheral blood lymphocytes, cells were solubilized using Triton X-100, and the radioactivity of the lysate was quantitated using a gamma counter. The specific labeling intensity of T cells was determined from control samples, and the proportion of added cells that adhered was then calculated. In some experiments, adhesion studies were performed in the presence of monoclonal antibodies directed against various endothelial cell and lymphocyte adhesion molecules. In these instances, OEC monolayers or T cells had been pretreated with medium containing these antibodies at previously determined optimum inhibitory concentrations (5–10 μg/ml) for 1 h prior to performing the adhesion assay.

Statistical analysis

Statistical significance was analyzed with a one-way analysis of variance. The result of correlation analysis (Pearson correlation coefficient) between cell counts derived from visual inspection and measurements of gamma emission has been reported previously (9). For determination of the interassay variability, the adhesion
assay was performed under identical baseline and stimulation conditions using OEC monolayers and T cells prepared on different days. Pair plotting analysis (day 1 versus day 2) revealed that all data points were within 11% of the line of identity, and 8/12 data points were within the ±5% line of identity.

### Results

**Isolation of microvascular OECs**

When tested on 11 orbital tissue samples (six obtained from patients with active, severe GO; five obtained from patients undergoing strabism surgery or enucleation for secondary glaucoma), the immunopurification technique described above had a success rate of > 85%, yielding highly purified OEC populations in sufficient cell numbers to permit formation of OEC monolayers in short-term tissue culture. The success rate of isolation and yield of OECs varied considerably depending on the size of the biopsy specimen, but was generally excellent for tissue sizes of > 0.8 cm². Adequate washing of beads and thorough mixing of cell suspensions between washes were found to be important steps in minimizing contamination with non-endothelial cells. In addition, performing two successive independent rounds of immunopurification using bead-immobilized monoclonal antibodies against distinct endothelial cell surface components also contributed significantly to high cell yield and purity. Compared to single-round immunopurification experiments (purity of 88 ± 9% for Ulex europaeus antigen I (UEA-I) and 82 ± 11% for anti-CD31 antibody), endothelial cell homogeneity was consistently > 98% when sequential immunopurification steps using both UEA-I and anti-CD31 antibody were performed. The OECs isolated by the combined immunopurification procedure formed typical cobblestone-like confluent monolayers within 5–7 days after isolation. No overgrowth by contaminating cells was noted.

**Phenotypical characterization of OECs**

Endothelial origin of the resulting cell population was suggested by the characteristic cobblestone-like appearance of OEC monolayers (Fig. 1A), and verified by

![Fig. 1](https://www.bioscientifica.com/)

*Fig. 1* Characterization of orbital endothelial cells (OECs) using phase-contrast microscopy and immunocytochemistry. Following immunomagnetic isolation and purification, OECs were plated on coverslips and processed for microscopic analysis and immunostaining using various monoclonal antibodies directed against endothelial cell-specific antigens. (A) Cobblestone-like morphology of unstained OECs. phase-contrast image; (B) OECs expressing immunoreactivity for thrombomodulin; (C) OECs expressing immunoreactivity for von Willebrand factor; (D) OECs expressing immunoreactivity for the endothelial cell antigen CD31. Original magnifications × 320.
detection of specific positive immunoreactivity when using antibodies directed against von Willebrand factor, thrombomodulin and human endothelial cells (CD31) (Fig. 1B–D). Negative immunostaining using monoclonal antibodies directed against cytokeratins, myosin, desmin, vitminen and fiblalost antigen further confirmed the homogeneity of the OEC population obtained (data not shown).

Expression of adhesion molecules in GO-OECs

Graves' ophthalmopathy OECs cultured under baseline conditions expressed ICAM-1 and CD44s (Fig. 2) (Table 1). Prior to stimulation, no immunoreactivity for ELAM-1 and only moderate degrees of VCAM-1 expression were detected (Table 1). Exposure of GO-OEC monolayers to TNF-α (100 U/ml), INF-γ (100 U/ml) and IL-1α (10 U/ml) markedly enhanced the level of ICAM-1 expression above baseline levels (p < 0.05) (Figs. 3 and 4; Table 1). By contrast, exposure of GO-OEC monolayers to IL-2 and TGF-β had no effect on ICAM-1 expression. Treatment with TNF-α and IL-1α, and to a lesser extent IFN-γ, significantly induced, in a dose-dependent manner, GO-OEC monolayers to express immunoreactivity for ELAM-1 and VCAM-1 (p < 0.05) (Fig. 4: Table 1).

Interactions between lymphocytes and GO-OECs

Minimal adhesion occurred when matched, unstimulated T cells were co-cultured with GO-OEC monolayers under baseline conditions. The percentage of adherent unstimulated T cells was increased significantly following exposure of GO-OECs to IL-1α and TNF-α (p < 0.05; data not shown). Activation of T cells with TPA further increased the percentage of cells attached to unstimulated GO-OECs. Adhesion was strongly enhanced when TPA-activated T lymphocytes were co-cultured with TNF-α-stimulated OEC monolayers (Fig. 5). Further, pretreatment of T cells with a monoclonal antibody specific for the hyaluronic acid binding site of CD44 (CD44(A)) significantly decreased adhesion of TPA-stimulated T-cell adhesion to activated GO-OECs (p < 0.05). Pretreatment of TNF-α-activated GO-OEC monolayers with hyaluronidase (250 μg/ml) also significantly decreased the extent of T-cell adhesion to activated OECs (p < 0.05). In contrast, pretreatment of T cells with a monoclonal antibody directed against an epitope of CD44 not involved in hyaluronic acid binding (Hermes III; CD44(B)) failed to significantly affect lymphocyte–GO-OEC interactions (Fig. 5).

The role of integrin-type adhesion receptors in mediating lymphocyte–GO-OEC binding was deter-

Table 1. Comparison of the effects of various forms of cytokine treatment on the expression of ICAM-1, ELAM-1, and VCAM-1 immunoreactivity in orbital endothelial cell (OEC) monolayers derived from patients with Graves' ophthalmopathy (GO) and normal individuals, respectively.*

<table>
<thead>
<tr>
<th>GO</th>
<th>ICAM-1</th>
<th>Control</th>
<th>GO</th>
<th>ELAM-1</th>
<th>Control</th>
<th>GO</th>
<th>VCAM-1</th>
<th>Control</th>
<th>GO</th>
<th>Control</th>
<th>CD44</th>
<th>Control</th>
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<td>Basal</td>
<td>17 ± 3*</td>
<td>2 ± 1</td>
<td>0</td>
<td>8 ± 2</td>
<td>0</td>
<td>17 ± 4*</td>
<td>3 ± 1</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α (100 U/ml)</td>
<td>63 ± 9*</td>
<td>34 ± 8</td>
<td>65 ± 11</td>
<td>58 ± 9</td>
<td>62 ± 8*</td>
<td>41 ± 7</td>
<td>35 ± 6*</td>
<td>4 ± 2</td>
<td></td>
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<tr>
<td>IFN-γ (10 U/ml)</td>
<td>47 ± 5*</td>
<td>8 ± 2</td>
<td>11 ± 6</td>
<td>4 ± 1</td>
<td>18 ± 4*</td>
<td>4 ± 3</td>
<td>18 ± 6*</td>
<td>3 ± 2</td>
<td></td>
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<tr>
<td>IL-1α (10 U/ml)</td>
<td>58 ± 7*</td>
<td>32 ± 9</td>
<td>57 ± 8*</td>
<td>34 ± 5</td>
<td>57 ± 7*</td>
<td>23 ± 6</td>
<td>61 ± 9*</td>
<td>14 ± 4</td>
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*Numbers indicate the percentages of cells expressing a particular adhesion molecule. Data represent the mean ± standard deviation derived from triplicate experiments, using OECs obtained from four patients with GO and four control individuals; p < 0.05 compared to control OECs.
mined using blocking monoclonal antibodies (Fig. 6). When applied alone, monoclonal antibodies directed against leukocyte function-associated antigen 1 (LFA-1) and very late antigen 4 (VLA-4), and their ligands ICAM-1 and VCAM-1, respectively, revealed partial inhibitory activity on the adhesion of activated T lymphocytes to GO-OECs stimulated by IL-1α or TNF-α (all \( p < 0.05 \)) (Fig. 6). In contrast, a monoclonal antibody specific for ELAM-1 failed to affect significantly the adhesion between activated T cells and GO-OECs. When administered simultaneously, monoclonal antibodies directed against ICAM-1 plus VCAM-1 and against LFA-1 plus VLA-4 were capable of inhibiting T-cell adhesion to activated GO-OECs to a greater extent than anti-LFA-1, anti-ICAM-1, anti-VCAM-1 or anti-VLA-4 antibodies applied alone (all \( p < 0.05 \)) (Fig. 6). Near-complete inhibition of T-cell adhesion was observed when monoclonal antibodies directed against ICAM-1, VCAM-1 and CD44 were administered together \( p < 0.01 \); (Fig. 6). In contrast, simultaneous addition of the anti-ELAM-1 monoclonal antibody failed to enhance significantly the inhibitory activity on T-cell adhesion already exerted by other monoclonal antibodies directed against ICAM-1, VCAM-1 or CD44 (Fig. 6). Taken together, these data indicate that integrin-dependent adhesion is important in T-cell binding to GO-OECs, and that CD44 also plays an important role in mediating integrin-independent adhesion of T lymphocytes to GO-OECs.

**Comparison between GO-OECs and control OECs**

Orbital endothelial cells derived from the retroorbital connective tissue of patients with untreated, active GO and control individuals were compared for their expression of adhesion molecules at baseline and following activation by various cytokines (Table 1). Under baseline conditions, GO-OECs expressed ICAM-1 and CD44 at low-to-moderate levels, while no expression was detected in control OECs. Immunoreactivity for ELAM-1 was absent in unstimulated OECs derived both from patients and control individuals, and low-grade VCAM-1 immunoreactivity was restricted to GO-OECs. Stimulation of GO-OECs and control OECs by IFN-γ, TNF-α and IL-1α resulted in varying degrees of expression for ICAM-1 and ELAM-1 (Table 1). The ICAM-1, ELAM-1 and VCAM-1 expression by GO-OECs was markedly enhanced by TNF-α and IL-1α, while IFN-γ showed only moderate effects. In control OECs. VCAM-1 expression was induced to a lesser degree by TNF-α and IL-1α, and no significant stimulatory effect was noted for IFN-γ (Table 1). In general, TNF-α, IL-1α induced expression of ICAM-1 and VCAM-1 in GO-OECs to a significantly greater extent compared with control OECs (all \( p < 0.05 \)). In addition, IL-1α induced ELAM-1 in a significantly greater percentage of GO-OECs.
Fig. 5. Effects of various forms of pretreatment on the adhesion of 12-0-tetradecanoyl-phorbol 13-acetate (TPA)-activated peripheral blood T lymphocytes to orbital endothelial cells (OECs) derived from patients with Graves' ophthalmopathy (solid bars) and normal individuals (open bars), respectively. The OECs were stimulated with TNF-α (100 U/ml). Where indicated, OECs were pretreated with a monoclonal antibody specific for the hyaluronic acid binding site of CD44 (CD44(A); at 10 μg/ml), a non-blocking monoclonal antibody specific for CD44 (CD44(B); at 10 μg/ml), or hyaluronidase (250 μg/ml), as indicated. Percentage adhesion was quantitated as described in the text. Bars indicate the percentages of lymphocytes adhering to unstimulated OECs and TNF-α-stimulated OECs without or with pretreatment with CD44 antibodies or hyaluronidase, as determined in a cell adhesion assay. Data represent the mean ± standard deviation of triplicate experiments, using OECs derived from three patients with GO and three control individuals, respectively: *p < 0.05 compared to basal + TNF-α.

Fig. 6. Effects of various adhesion molecule-specific monoclonal antibodies on the adhesion of peripheral blood T lymphocytes to orbital endothelial cells (OECs). 12-0-Tetradecanoyl-phorbol-13-acetate (TPA)-activated lymphocytes were pretreated with various combinations of monoclonal antibodies specific for LFA-1, VLA-4 and CD44 (each at 10 μg/ml), respectively, as indicated. The OECs were stimulated with TNF-α (100 U/ml) and pretreated with monoclonal antibodies specific for ICAM-1, ELAM-1, VCAM-1 and CD44 (each at 10 μg/ml), respectively, as indicated. Percentage adhesion was quantitated as described in the text. Bars indicate the percentages of lymphocytes adhering to OECs following pretreatment with mouse control IgG and specific monoclonal antibodies, as determined in a cell adhesion assay. Data represent the mean ± standard deviation of triplicate experiments, using OECs derived from three patients with Graves’ ophthalmopathy: *p < 0.05 and **p < 0.01 compared to non-immune mouse IgG control, respectively.

Fig. 7. Adhesion of unstimulated and 12-0-tetradecanoyl-phorbol 13-acetate (TPA)-stimulated matched peripheral blood T lymphocytes to unstimulated Graves’ ophthalmopathy orbital endothelial cells (GO-OECs: solid bars) and normal OECs (open bars), respectively. Percentage adhesion was quantitated as described in the text. Bars indicate the percentages of lymphocytes adhering to OECs, as determined in a cell adhesion assay. Data represent the mean ± standard deviation of triplicate experiments, using OECs derived from three patients with GO and three control individuals: *p < 0.05 compared to unstimulated T cells.

Compared to control OECs (p < 0.05). Tumor necrosis factor α and, in particular, IL-1α stimulated expression of CD44 to a significantly greater extent in GO-OECs compared with control OECs (p < 0.05) (Table 1). Further, unstimulated T cells failed to adhere to either unstimulated GO-OECs or control OECs. In contrast, TPA activation of T cells resulted in greater enhancement of cell adhesion to unstimulated GO-OECs compared with control OECs (Fig. 7). Further, major differences in T-cell adhesion to untreated or cytokine-activated GO-OECs and control OECs following administration of blocking monoclonal antibodies directed against various adhesion molecules were noted. For example, following preincubation with monoclonal antibodies directed against ICAM-1, VCAM-1 and CD44, adhesion of TPA-activated cells to unstimulated GO-OECs was inhibited to a greater extent compared to control OECs (data not shown). Similarly, following stimulation of OECs by TNF-α or IL-1α, TPA-activated T cells adhered to GO-OECs in significantly greater numbers compared to control OECs (p < 0.05). Further, treatment with anti-VCAM-1 monoclonal antibody inhibited T-cell adhesion to GO-OECs to a significantly greater extent compared with control OECs (p < 0.01). Finally, in contrast to control OECs, adhesion of activated T cells to GO-OECs was inhibited markedly by pretreatment of T cells with a monoclonal antibody specific for the hyaluronic acid binding site of CD44 (anti-CD44(A)), or by treatment of activated control OEC monolayers with hyaluronidase, but not...
by pretreatment of T cells with a monoclonal antibody specific for a CD44-epitope not involved in HA binding (anti-CD44(B)) (Fig. 5).

Discussion

Recent experiments from several laboratories suggest an important role for T lymphocytes in the pathogenesis and propagation of GO. De Carl et al. demonstrated that retrobulbar T cells cultured in vitro release a T helper 1 (Th-1)-like cytokine profile and exhibit non-specific cytotoxicity (13). Grubeck-Loebenstein and colleagues have shown that retrobulbar CD8+ T cells are capable of specifically recognizing autologous retrobulbar fibroblasts without necessarily exerting target cell cytotoxicity (12). Moreover, we have demonstrated recently a marked restriction of the T-cell antigen receptor variable-region (TcR V) gene repertoire in T cells infiltrating the retroorbital space of patients with severe inflammatory GO of short duration (17). In contrast, greater diversity of the TcR V gene repertoire was present in patients studied during later stages of GO, and unrestricted TcR V gene usage or loss of detectable TcR-specific mRNA was detected in patients undergoing rehabilitative eye muscle surgery during late, inactive stages of their disease. Thus, T-cell recognition of retroorbital antigens appears to play an important role in the pathogenesis of GO. Although the nature of such antigenic structures has yet remained poorly defined, candidate antigens such as eye muscle and fibroblast antigens and the human thyrotropin receptor or variants thereof have been proposed (2, 11, 18, 19).

Transendothelial migration of lymphocytes into the extravascular retroorbital space depends upon the expression of a finely tuned cascade of cytokine-activated adhesion receptors by endothelial cells of the retroorbital microvasculature. Using cell populations involved in vivo in lymphocyte recruitment to the orbit in GO, we have begun to study in vitro the attachment of circulating T lymphocytes to OECs. Following two rounds of immunomagnetic purification with monoclonal antibodies directed at distinct vascular cell antigens, highly homogenous populations of OECs were isolated from small retroorbital tissue specimens and characterized by immunostaining with various endothelial cell-specific monoclonal antibodies. Cell numbers of OECs obtained by this method were sufficient to permit OEC monolayer formation and propagation in vitro for at least three cell passages.

Adhesion receptors expressed by cytokine-activated microvascular endothelial cells were identified using immunocytochemical staining and their functional activity determined by blocking experiments in a cell adhesion assay. Our results suggest that ligand/receptor interactions involving both ICAM-1/LFA-1- and VCAM-1/VLA-4-mediated adhesion pathways represent important mechanisms by which T lymphocytes can bind to OECs in vitro. Activation by cytokines induced OECs to express immunoreactivity for ELAM-1 and VCAM-1, and increased the percentage of ICAM-1-positive OECs. However, as demonstrated by blocking experiments, binding of activated T lymphocytes to stimulated OECs did not appear to involve ELAM-1, but was partially dependent on interactions involving ICAM-1 and VCAM-1 expressed by OECs, and the integrins LFA-1 and VLA-4 expressed by activated lymphocytes, respectively.

An important result of this study was the detection of integrin-independent adhesion of T lymphocytes to GO-OECs as mediated by a hyaluronic acid-binding epitope of CD44; CD44 is a glycoprotein with homology to core and link proteins and a receptor for hyaluronic acid, and has been shown to serve as a homing molecule (20, 21); CD44 is expressed on a wide variety of cells, including fibroblasts and activated lymphocytes (20). Engagement of other adhesion receptors may activate CD44 on lymphocytes, thereby enhancing their affinity for hyaluronic acid (23, 24). T-cell adhesion is accompanied by the clustering of CD44 molecules on the surface of lymphocytes oriented toward the endothelium (22, 25). Within the retroorbital space in GO, CD44 has been localized to connective tissue fibroblasts, vascular endothelium and infiltrating mononuclear cells (26). Expression of CD44 on GO-OECs as well as on activated T lymphocytes, and the ability of hyaluronidase and a CD44 monoclonal antibody to disrupt the lymphocyte–GO-OEC interaction, suggest that CD44 on T lymphocytes binds to GO-OEC-associated hyaluronic acid and that hyaluronic acid possibly provides a bridge between these two CD44-positive cell populations. Our demonstration that CD44 can act as an important mediator of integrin-independent T-lymphocyte adhesion to GO-OECs emphasizes the potential relevance of CD44 and hyaluronic acid in T-lymphocyte trafficking. This may be particularly relevant in an inflammatory environment such as the retroorbital space in GO, where CD44 is expressed by various cellular components and where hyaluronic acid is present in abundant quantities (26).

Although our data appear to support the concept that particular adhesion molecules expressed by GO-OECs may be involved in orbit-specific lymphocyte recruitment, it must be remembered that these experiments are based on studies conducted in vitro and thus may be subject to influences that may arise during culture of effector and target cells ex vivo. However, several lines of histological and functional evidence suggest that lymphocyte interactions with GO-OECs are likely to occur in vivo and may be intimately involved in the evolution of GO. Intercellular adhesion molecule-1, a ligand of LFA-1, and VCAM-1, a ligand of the integrin receptor VLA-4, are expressed by fibroblasts, vascular endothelium and postcapillary venules within the retroorbital tissues in GO (7). In addition, LFA-1 was
found to be expressed by lymphocytes and monocytes infiltrating the retroorbital space in GO. Further, expression of ICAM-1 and VCAM-1 immunoreactivity by retroorbital fibroblasts has been detected both in vitro and within the retroorbital connective tissue of patients with active GO (7, 9). This suggests that lymphocytes expressing LFA-1 and VLA-4 may interact with retroorbital fibroblasts via ICAM-1 and VCAM-1. Both ICAM-1 and VCAM-1 are induced by proinflammatory cytokines, namely IFN-γ, TNF-α and IL-1, on retroorbital fibroblasts (9) and, as demonstrated here, on orbital endothelial cells. Immunoreactivity for these cytokines has been detected in situ within the retroorbital tissues in GO (6). In particular, TNF-α immunoreactivity was localized specifically to vascular endothelial cells within specimens derived from the retroorbital connective tissue of patients with active GO, but not healthy individuals. Further, analysis of cytokines in fresh GO retroorbital tissue explants, cultured fibroblasts and supernatants derived from these experiments suggested that IFN-γ and TNF-α are produced by infiltrating lymphocytes and macrophages, while IL-1α may also be derived from resident fibroblasts (6). As demonstrated here, the spectrum of adhesion molecules expressed by GO-OECs at baseline and following activation by cytokines revealed a number of differences compared with OECs derived from control individuals. Thus, local expression of certain adhesion molecules following appropriate stimulation of OECs by cytokines and possibly other factors (9) may be a critical step in the initiation and perpetuation of lymphocyte recruitment to the retroorbital space in GO. To examine further this hypothesis, a series of adhesion studies are currently under way using retroorbital lymphocytes and endothelial cells derived from other involved and non-involved extrathyroidal sites of patients with Graves’ disease. Interestingly, both the ICAM-1/LFA-1 and the VCAM-1/VLA-4 adhesion pathways appear to be involved in T-lymphocyte binding to vascular endothelium in Graves’ and Hashimoto’s thyroid glands (27) and in various other conditions of autoimmunity directed against connective tissue cells (14, 28–30).

In conclusion, highly pure populations of OEC can be isolated from small volumes of orbital connective tissue and propagated in vitro. Analysis of adhesion molecules expressed by these cells, their differential regulation and their functional contributions in lymphocyte–OEC interactions suggest that site-directed recruitment of T lymphocytes in GO involves both integrin-independent and integrin-independent mechanisms. Further analysis of lymphocyte–OEC interactions will help to elucidate the potential benefits of therapeutic strategies designed to block lymphocyte access to the retroorbital space at the level of the endothelial cell.

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