Expression of Functional CD40 by Vascular Endothelial Cells

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Summary

The interaction between activated vascular endothelium and T cells has been shown to play an important role in the recruitment and activation of T cells at sites of inflammation. Here we report the expression of CD40 by vascular endothelial cells and its regulation by inflammatory agents. Using the soluble recombinant CD40 ligand, gp39, we show that the interaction of CD40 with its ligand can lead to endothelial cell activation, which in turn leads to leukocyte adhesion. This adhesion is partly mediated by the expression of E-selectin. In addition to E-selectin expression, gp39 induces the expression of intercellular adhesion molecule 1 and augments the tumor necrosis factor α-induced expression of vascular cell adhesion molecule 1. The effects of gp39 on endothelial cells can be blocked with anti-gp39 monoclonal antibody (mAb), anti-CD40 mAb, or soluble CD40. Staining of tissues from healthy human skin using anti-CD40 mAb showed very weak expression of CD40 by the endothelium, while skin involved in inflammatory disease showed marked upregulation of CD40 expression. These studies suggest that interactions between cell surface proteins expressed by activated T cells with their receptors on vascular endothelium can stimulate the vasculature at sites of inflammation and may be involved in normal inflammatory responses and in inflammatory disease.

CD40, a 50-kD glycoprotein expressed by a range of cell types of the immune system, functions as a signaling protein. It was initially described and studied on B cells, where stimulation with anti-CD40 mAb was shown to induce proliferation and isotype switching in the presence of an appropriate costimuli. For example, early studies demonstrated that anti-CD40 mAbs are synergistic with PMA, anti-CD20 mAb, or IL-4 for the induction of B cell proliferation (1-4). Further studies have shown that anti-CD40 treatment with IL-4 as the costimulator results in the secretion of IgE, IgM, IgA, and soluble CD23 (4-8). With IL-10 as the costimulator, anti-CD40 treatment results in the secretion of IgA, IgM, and IgG (9, 10). In addition, anti-CD40 presented by CD32-transfected L cells in combination with IL-10 and TGF-β results in the production of IgA from sIgD+ B cells (9). CD40 is also expressed and functional on several other cell types. Monocytes respond to anti-CD40 or CD40 ligand (gp39) in conjunction with GM-CSF, IL-3, or IFN-γ by producing cytokines (11). Thymic epithelium has also been shown to express CD40, and stimulation with anti-CD40 in conjunction with IFN-γ and IL-1 leads to secretion of GM-CSF (12). CD40 is expressed by normal basal epithelium, follicular dendritic cells, and some carcinoma- and melanoma-derived cell lines (13-16). More recently, it has been found that T cells express CD40 and respond to gp39 by the proliferation and expression of cytokines and activation markers (17), and dendritic Langerhans cells respond to ligation of CD40 by altering their morphology and surface molecule and cytokine expression (18).

The molecular steps leading to the recruitment and activation of leukocytes at sites of inflammation are being dissected. The current hypothesis is that leukocyte recruitment and activation at sites of inflammation is a sequential multistep process (for review see references 19, 20). First, adhesion proteins of the selectin family mediate the rolling of leukocytes on the activated vascular endothelial cells. The rolling leukocytes then probe the endothelial cell surface and come into contact with high local concentrations of cytokines and chemokines, which emanate from the site of inflammation and are bound and presented by proteoglycans on the endothelial cell surface. These proinflammatory molecules activate the leukocytes, which then become firmly attached to the vascular cell wall, a process thought to be primarily mediated by members of the integrin family of receptors. The firmly bound leukocytes then extravasate into the surrounding tissue and migrate to the site of inflammation. The role of endothelial cell surface proteins in this process has been the subject of intensive study. For example, expression of P-selectin (CD62P),
E-selectin (CD62E), intercellular adhesion molecule 1 (ICAM-1); (CD54), and vascular cell adhesion molecule 1 (VCAM-1) (CD106) by activated endothelial cells results in leukocyte binding to the activated endothelial cells. Furthermore, the interaction of molecules such as ICAM-1 and VCAM-1 with inflammation these cells may also present antigen (21).

There is evidence that the cell surface proteins expressed by some of the activated leukocytes transiently bound to the endothelial cells at sites of inflammation provide stimulatory signals to the endothelial cells. For example, studies have shown that activated T cells can alter the expression of MHC or enhance vascular permeability in a cell contact–dependent fashion (22, 23). Thus, signals delivered to the endothelial cells by cell surface proteins that are expressed by activated leukocytes may play an important role in ensuring an effective inflammatory response. These interactions may enhance the ability of the endothelial cell to recruit and activate leukocytes, facilitate the molecular events that lead to leukocyte diapedesis, and/or enhance the ability of the endothelial cells to act as APC.

To investigate if the T cell activation antigen gp39 (CD40L, TRAP, T-BAM) is involved in the stimulation of vascular endothelial cells during endothelial cell–T cell interactions at a site of inflammation, we examined if vascular endothelial cells express CD40, how its expression might be regulated, and if ligation of CD40 results in endothelial cell activation.

Materials and Methods

Antibodies and Reagents. The mAb against CD40 (G28-5, mouse IgG1), a gift from J. Ledbetter (Bristol-Myers Squibb, Seattle, WA), has been described previously (1). TNF-α produced in Escherichia coli was purchased from R&D Systems, Inc. (Minneapolis, MN), recombinant IL-1β produced in E. coli, and IFN-γ were purchased from Genzyme Corp. (Cambridge, MA); LPS, isolated from E. coli O111:B4 and phenol/water purified, was purchased from Sigma Chemical Co. (St. Louis, MO); gp39, a fusion protein consisting of the extracellular domain of murine CD8 (Ly2) and the extracellular domain of gp39, was produced by transient expression in COS cells as previously described (24) and purified by chromatography on an anti-Ly2 affinity column. mAb 39-1.106 was a gift from A. Ol11:B4 and phenol/water purified, was purchased from Sigma Chemical Co., Redmond, WA; 100 μl per well, 1:100 dilution) and stopped with 100 μl/well of 1 N H2SO4. The absorbance was determined at dual wave lengths of 450 and 630 nm.

PMN/Endothelial Cell and HL60/Endothelial Cell Adhesion Assays. Adhesion of PMN cells or the premeloyctic cell line HL-60 to endothelial cells was measured essentially as described, with modifications (25). Briefly, PMN were separated from whole blood with Polymorphoprep™ (Nycomed), resuspended to a density of 4 × 106 cells per ml in PBS containing 1 mM each of CaCl2 and MgCl2, and 10 mM 2,7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein, ace toxy methyl ester (Molecular Probes, Inc., Eugene, OR), and then incubated in the dark for 15 min at 37°C. An equal volume of RPMI media was added, and the cells were pelleted and washed with 1 mM CaCl2/MgCl2 PBS and resuspended at 2.5 × 106 cells per ml. Labeled PMN, 100 μl per well, were added to a monolayer of HUVEC or HAEC that had been plated as described above and activated for 4 h. Plates were swirled gently at room temperature for 30 min in the dark, the wells were washed twice with PBS, and the adherent neutrophils were lysed by the addition of 100 μl per well of 1% SDS in 50 mM Tris, pH 8. Fluorescence was measured on a microplate fluorescence reader. Assays for the adherence of HL-60 cells were performed using the same procedure.

Patients. Patients with allergic contact dermatitis (n = 5) and
psoriasis (n = 4) were compared with healthy control subjects (n = 5). None of the patients had received previous treatment for their skin condition. After we obtained informed consent, full-thickness 4-mm punch biopsies were taken from the lesional skin of the patients. In the control group, 4-mm punch biopsies were taken from the volar aspects of the forearm. Samples were snap frozen immediately and stored at -80°C until use.

**Immunohistochemistry.** Frozen skin specimens were embedded in OCT compound (Miles Inc., West Haven, CT), and 5-μm serial cryostat sections were prepared using a cryostat (Crycut 2000; Reichert Jung, Nussloch, Germany). Air-dried, acetone-fixed frozen sections were stained using a four-step immunohistochemical staining protocol as described previously (27). Briefly, cells were stained by sequential treatment with (a) primary mAb (G28-5, mouse IgG1), (b) biotin-conjugated goat anti-mouse IgG, (c) peroxidase-conjugated streptavidin, and (d) diaminobenzidine as chromogenic substrate. Finally, sections were counterstained with hematoxylin. Control stainings were performed by replacing the primary mAb with isotype-matched control reagents. The stained samples were evaluated by four independent observers in a blinded fashion using an Axioskop equipped with a MC100 camera system (Carl Zeiss, Inc., Thornwood, NY).

**Results**

**Endothelial Cells Express CD40.** To examine if CD40/gp39 binding may play a role in the interaction between activated T cells and activated endothelial cells, we first examined whether CD40 is expressed on endothelial cells. HUVEC of passage number 5 were stained with anti-CD40 mAb or gp39 and examined by flow cytometry. As shown in Fig. 1, CD40 is expressed by resting endothelial cells in culture. HAEc were also found to express CD40, as detected by FACS® analysis (data not shown). To examine if this basal level of CD40 expression can be altered by proinflammatory mediators, HUVEC were incubated with TNF, IFN-γ, LPS, or IL-1β. After 24 h, cells treated with the inflammatory cytokines or LPS all showed slight but significant increases in expression of CD40 (Fig. 2). Similar effects were seen after stimulation for 72 h (data not shown). These results suggest that the expression of CD40 by endothelial cells can be regulated by agents known to play a role in inflammation. Additionally, it was found that treatment of the cells with an anti-CD40 antibody for 24 h substantially reduced the level of CD40 detected on the surface of the cells. In these experiments, the cells were incubated with the G28-5 antibody, and then subsequently stained with additional G28-5 antibody. In this way, the total G28-5 bound to the cell surface is detected. The reduced level of CD40 detected is likely to be caused by internalization of the CD40. This possibility is suggested by the observation that a G28-5 immunotoxin was capable of killing CD40-expressing B cell lymphomas, a process dependent on internalization (15).

**Leukocyte Binding to Endothelial Cells Can Be Stimulated via CD40.** The ability of endothelial cells to respond to ligand binding...
Figure 2. Regulation of CD40 expression on endothelial cells by inflammatory mediators and anti-CD40 mAb. HUVEC were treated with the indicated agent for 24 h, fixed, and assayed for CD40 expression by ELISA as described in Materials and Methods. Value is the mean of three points.

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CD40 Stimulation Leads to E-Selectin Expression. To determine the molecular basis of the gp39-induced leukocyte-HUVEC cell adhesion, we examined the levels of E-selectin expression after gp39 stimulation. As shown in Fig. 4, incubation of HUVEC with gp39 resulted in the expression of significant levels of E-selectin. The ability of gp39 to induce E-selectin expression was completely inhibited by the addition of anti-gp39 mAb. To investigate whether other types of endothelial cells were able to respond to ligation of CD40, healthy HAEC were also tested for the ability to express E-selectin after stimulation with gp39. Like the HUVEC, the HAEC expressed E-selectin in response to gp39 in a concentration-dependent manner. This induction was inhibited by a blocking antibody to gp39 (Fig. 4 B). Incubation of HUVEC with the control fusion protein, CD72, did not drive E-selectin expression (data not shown). The ability of gp39 to induce E-selectin expression was also inhibited by CD40Ig, a fusion protein consisting of the extracellular domain of CD40 fused to human Ig (24) and the anti-CD40 antibody G28-5. The G28-5 antibody was unable to stimulate E-selectin expression. These results are similar to that seen by Lederman et al. (28), where anti-CD40 was used to inhibit the ability of gp39-expressing cells to stimulate CD23 expression by B cells. Combinations of suboptimal levels of TNF and gp39 were additive and not synergistic in E-selectin induction (data not shown).

Stimulation through CD40 Leads to Expression of ICAM-1 but Not VCAM-1. To investigate the role of gp39 in the induction of expression of other cell adhesion molecules, the expression of ICAM-1 and VCAM-1 was measured after 24 h of stimulation with gp39 (Fig. 5). Expression of ICAM-1 was induced by gp39 in a concentration-dependent manner. This induction was inhibited by the F(ab')2 fragment of an anti-gp39 antibody, 39-1.106. In contrast, VCAM-1 was not induced by gp39. However, the induction of VCAM-1 by low levels of TNF was augmented by gp39 in a concentration-dependent manner. This increase was blocked by the anti-gp39 mAb F(ab')2.

Expression of CD40 by Endothelial Cells in Tissue Sections. We examined the expression of CD40 in vivo by immunohistochemistry. In healthy human skin, little if any CD40 was detected on dermal endothelial cells (Fig. 6 A). By contrast, a marked upregulation of endothelial cell CD40 expression was a constant feature of inflammatory skin disease, such as allergic contact dermatitis and psoriasis (Fig. 6, B and C). Furthermore, in psoriasis, an enhanced expression of CD40 was detected on keratinocytes in the basal and suprabasal layers, as well as on the dermal mononuclear infiltrate (Fig. 6, B and C). The detection of CD40 on HUVEC in culture, but not in healthy skin, may be a reflection of the partially activated state of the HUVEC under the culture conditions used. This has been observed previously, for example, in the expression of CD44, where slightly different conditions used in different laboratories yielded differing levels of expression. The ability to further increase CD40 expression indicates the ability of the cells to respond despite the "inflamed" characteristics of the cultured cells (29, 30).
Discussion

CD40 is known to be a signaling receptor for a range of cell types of the immune system. Here we report the expression of functional CD40 by endothelial cells, the increase of CD40 expression in inflammatory skin disease, and a possible role of a CD40-gp39 interaction in the regulation of cell adhesion molecules. Endothelial cells are known to play a role in the initiation of inflammation. Inflammatory mediators induce endothelial cell expression of adhesion molecules to initiate the recruitment of leukocytes to the inflamed area. In this process, the leukocytes are stimulated by proteins expressed by, or presented on, the activated endothelium, including proteoglycan bound cytokines and surface proteins such as the integrin ligands ICAM-1 or VCAM-1 (31–34).

The endothelium is also stimulated by the migrating leukocytes, thus providing a mechanism for amplification and extension of the inflammatory response. In addition to playing a key role in the recruitment of leukocytes during inflammation, endothelial cells may express MHC class I and MHC class II, thus allowing the endothelial cell to function directly as an APC (35). The finding reported here, that endothelial cells express CD40, a receptor present on monocytes, B cells, and dendritic cells, strengthens the notion of these cells as APC and provides a mechanism for contact-dependent signaling of the endothelial cell by the migrating leukocyte.

We have demonstrated that signaling through CD40 induces the expression of adhesion molecules on the endothelial

Figure 4. E-selectin expression on endothelial cells induced by gp39. (A) HUVEC and (B) HAEC were treated with gp39 for 4 h in the presence (solid triangles) or absence (open squares) of an anti-gp39-blocking mAb, 39-1.106 (5 µg/ml), then tested for the expression of E-selectin by ELISA. Values are the mean of three points, and error bars represent the SD. Error bars are absent when the bar is smaller than the graph symbol.
cell, thus providing a possible mechanism for the reported ability of primed CD4\(^+\) T cells to stimulate endothelial cell adhesion molecule expression (36). This is similar to the induction of adhesion in CD40-stimulated B cells, where a homotypic aggregation occurs in culture in response to anti-CD40 by the induction of ICAM-1 as well as the activation of the CD18 integrins (37). In the case of endothelial cell–activated T cell interactions, it might be expected that the integrins would be present in a high-avidity form, and the induction of ICAM-1 allows for a high-avidity heterotypic interaction. These interactions have been shown to be required for intercellular signaling and diapedesis (38). For example, the induction of proliferation of B cells in response to in vitro–activated T cells can be blocked by anti-CD18 mAb (39). The tight adhesion provided by the integrin–integrin ligand may allow contact for T cell–endothelial cell signaling, leading to extension or amplification of the inflammation and/or migration of the bound T cell. The differential effect of CD40 signaling on the expression of ICAM-1 versus VCAM-1 also suggests that the gp39–CD40 system may contribute to the specificity of the cell populations that are recruited by the endothelium by altering the pattern of adhesion molecules that are expressed. Evaluation of the role of these adhesion molecules for the interaction of endothelial cells with other gp39-expressing cell types awaits identification of the requirements of activation for gp39 expression and nature of the coexpressed adhesion molecules.

The ability of gp39-expressing cells to stimulate endothelial cells has implications in several disease states. Here we report that gp39 induces endothelial cell expression of adhesion proteins involved in the initiation and maintenance of an inflammatory response. In a normal inflammatory response, a process to limit these mechanisms and to allow for a resolution of the inflammation is expected. However, gp39 may play a role in inflammatory disease states, where aberrant expression may maintain activation. Our finding that CD40 is expressed on endothelial cells in inflammatory skin disease demonstrates an in vivo association of this receptor with inflammation. Functional CD40–gp39 interaction may play a role in other forms of inflammation and disease, such as chronic graft rejection, where the stimulation of the endothelial cells by host lymphocytes induces mononuclear cell infiltrate, vascular smooth muscle cell activation and proliferation, and ECM deposition. In acute rejection, the allogeneic stimulation of host T cells would be expected to induce expression of gp39 along with other stimulatory proteins, inducing expression of adhesion molecules on the graft endothelium. Indeed, expression of VCAM-1 and ICAM-1 are indicative of rejection and precede leukocyte infiltration (40–42). The presence of a population of T cells that are responsive to endothelial cells has also been indicated in autoimmune states. For example, induction of autoimmunity in insulin-dependent diabetes mellitus in rats correlates with the presence of T cells that are able to activate the endothelium (43). This suggests the importance of this interaction in vivo and the potential role of CD40–gp39 interactions in autoimmunity.

In summary, we have identified the expression of CD40 by activated endothelial cells and have demonstrated the ability to induce the expression of adhesion proteins through this receptor. The induction of CD40 in allergic contact dermatitis and psoriasis suggests an association of CD40 expression with inflammatory disease that is supported by the ability of inflammatory mediators to upregulate expression in vitro. The consideration of endothelial cells as APC is also supported by the expression of functional CD40; this is the subject of further study.

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