Costimulator Deficient Antigen Presentation by an Endothelial Cell Line Induces a Nonproliferative T Cell Activation Response without Anergy

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Summary

The ability of endothelial cells to activate helper T (Th) cells by antigen presentation was studied using the murine endothelial cell line SVEC4-10 and antigen-specific murine T cell clones. SVEC4-10 cells constitutively express vascular cell adhesion molecule 1 but not intercellular adhesion molecule 1. Interferon γ (IFN-γ) treatment of these cells induced class II major histocompatibility complex (MHC) expression and antigen-presenting capabilities, but did not alter surface integrin expression. IFN-γ–treated SVEC4-10 cells were competent at mediating antigen-dependent cytokine production and proliferation of a Th2 clone. In contrast, endothelial antigen presentation to Th1 cells did not stimulate T cell proliferation. The addition of MHC mismatched spleen cells as a source of costimulatory molecules resulted in the ability of the endothelial cells to stimulate Th1 cell proliferation in an antigen-specific manner. The failure of the endothelial cell line alone to support Th1 cell proliferation correlated with the failure to stimulate interleukin 2 (IL-2) gene expression. T cell exposure to the endothelial cells plus antigen resulted in upregulation of IL-2 receptors and an enhanced response to subsequent antigen presentation by splenic antigen-presenting cells. Despite the lack of functional costimulators for IL-2 expression, antigen presentation by the endothelial cell line did not induce Th1 cell anergy, indicating that costimulator deficiency for IL-2 expression is not obligatorily linked to anergy induction. Thus, endothelial cells are capable of presenting antigens to helper T lymphocytes, but stimulate only partial T cell responses. These partial responses may serve to selectively stimulate transmigration of antigen-specific T cells and may enhance functional responses upon subsequent, extravascular antigen exposure.
specific for exogenous protein antigens (21). The presence or absence of endothelial costimulators for distinct subsets of differentiated helper T cells has not been examined.

The presence or absence of costimulators on APCs may profoundly influence the outcome of T cell antigen recognition. With costimulators present, antigen presentation results in IL-2 gene expression, T cell proliferation, and the generation of T cell progeny capable of responding to subsequent antigen exposure. In contrast, several experimental models have suggested that antigen presentation in the absence of costimulators causes suboptimal or no IL-2 gene expression (22, 23). Furthermore, costimulator-deficient APC may induce a long-lived anergic state in which the T cell cannot transcribe its IL-2 genes in response to subsequent antigen recognition (22-28). This phenomenon of clonal anergy has largely been studied using murine Th1 clones together with APCs that are chemically fixed to destroy mostly uncharacterized costimulatory activities (27). There is evidence that some unfixed cell types, such as keratinocytes (29) and renal tubular epithelial cells (30) may induce anergy, presumably because of the lack of expression of costimulators. Furthermore, T cell anergy has been induced by T cell receptor stimulation in the complete absence of accessory cell costimulators using peptide bound to MHC molecules on planar membranes (31), or immobilized antireceptor antibodies (32).

In this paper, we describe the antigen-presenting functions of a well differentiated murine endothelial cell line, SVEC4-10 (13). Our findings indicate that these endothelial cells have the capacity to stimulate proliferation of some helper T cells but not others. Furthermore, we show that the inability to stimulate some T cells is due to a lack of costimulators, which can be supplemented by the addition of nonantigen-presenting allogeneic spleen cells. Importantly, these viable, costimulator-deficient endothelial cells do not induce anergy in the A.E7 T cell clone that has been used as a prototype for anergy induction by chemically fixed APCs (26-28, 33-37). The findings are discussed in the context of a hypothetical role of endothelial antigen presentation to circulating T cells.

Materials and Methods

Mice. BALB/cJ and AKR mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell Lines. The endothelial cell line SV4EC-10 (13) derived from a C3H/HeJ mouse lymph node was obtained from Dr. Michael Edelin (Johns Hopkins University, Baltimore, MD), and grown in DME with 10% FCS. Some SVEC4-10 cultures were treated with 100 U/ml recombinant murine IFN-γ (Genzyme Corp., Cambridge, MA) for 6 d before use in functional assays to induce class II MHC expression. The mouse CD4+ helper T cell clones used in these studies included: A.E7, specific for pigeon cytochrome C + I-Ek, a gift of Dr. Ronald Schwartz (National Institutes of Health, Bethesda, MD); 100.9, specific for rabbit γ globulin + I-Ak, a gift of Dr. Abul Abbas (Brigham and Women’s Hospital, Boston, MA); and D10.G4, specific for conalbumin + I-Ak, obtained from the American Type Culture Collection (Rockville, MD). The clones were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and 5 × 10^-3 M 2-mercaptoethanol. The clones were stimulated with 100 μg/ml antigen plus 1,500 rad irradiated whole AKR spleen cells every 4 d. Recombinant murine IL-2 (10 U/ml) or supernatant from Con A-stimulated rat spleen cells (10% vol/vol) was added 4 d after stimulation. Pigeon cytochrome c (PCC), 1 conalbumin, and rabbit γ globulin were obtained from Sigma Chemical Co. (St. Louis, MO). The 81-104 peptide of the PCC molecule, recognized by the A.E7 clone was a gift from Dr. Abul Abbas. Cloned T cells were harvested between 10 and 14 d after restimulation for experimental cultures, and viable T cells were isolated by centrifugation over Ficoll-Diatrizoate (Organon Teknika, Durham, NC).

Mouse recombinant IL-2 was used as a culture supernatant from the x63-IL-2 cell line that constitutively expresses a transfected murine IL-2 gene, generously provided by Dr. Fritz Melchers (Basil Institute for Immunology, Switzerland) (38). Bioactivity of recombinant IL-2 preparations was determined using the HT-2 indicator line, as described below. One unit of IL-2 was defined as the amount required to produce half-maximal proliferation of the indicator cell line in a serial dilution assay.

Antigen Presentation Assays. Untreated or IFN-γ-treated SVEC4-10 cells were grown to confluence in gelatin-coated flat bottom 0.2-ml microtiter wells. The monolayers were treated with 50 μg/ml mitomycin C (Sigma Chemical Co.) for 90 min at 37°C, washed, and 4 × 10^4 cloned T cells were added with or without antigen. T cell proliferation was assayed by labeling with [3H]TdR (Amersham Corp., Arlington Heights, IL) (1 μCi/well) for the final 6 h of a 3-d culture. Incorporate radioactivity was measured in a Betaplate scintillation counter (LK-B-Pharmacia, Gaithersburg, MD). To measure secretion of IL-2, 50-μl aliquots of supernatant were collected from each microwell 18 h after initiation of the culture and these were assayed for their ability to stimulate growth of the HT-2 indicator cells line (39).

Assay for Anergy Induction. For the attempted induction of anergy, 5 × 10^4 A.E7 T cells were cocultivated on IFN-γ-treated SVEC4-10 monolayers in 2-ml wells for 24 h in the absence or presence of 10 μM PCC peptide fragment 81-104 or 100 μM intact PCC. The T cells were then removed from the monolayers and restaked in tissue culture medium alone for either 48 h or 7 d. After rest, they were recultured in 0.2-ml wells at 2 × 10^6 viable cells per well and restimulated with irradiated AKR spleen cells plus PCC peptide or PCC as described above, or 10 U/ml recombinant murine IL-2 and T cell proliferation was measured at 48 h. As a positive control for anergy induction, primary cultures were also set up with 5 × 10^4 A.E7 T cells and 2 × 10^6 AKR spleen cells that had been previously fixed with 50 mM ECDI as previously described (26) followed by rest and restimulation cultures identical to those described above.

Immunofluorescence Assays. Immunofluorescent staining of SVEC4-10 cells was performed on single-cell suspensions prepared from subconfluent monolayers by EDTA treatment. The cells were incubated with primary unlabeled rat anti-mouse mAbs of the appropriate specificities at 4°C for 30 min, washed in ice-cold serum-free tissue culture medium, and incubated for an additional 30 min at 4°C with FITC-labeled goat anti-rat Ig (Southern Biotechnology Associates, Birmingham, AL). The cells were then washed in ice-cold PBS, fixed in 1% paraformaldehyde, and analyzed by flow cytometry on a FACScan instrument (Becton Dickinson & Co., Mountain View, CA). T lymphocytes that were cocultured on

1 Abbreviations used in this paper: ECDI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl; PCC, pigeon cytochrome c.
SVEC4-10 monolayers for 24 h were harvested by gentle pipetting and stained in the same manner.

**Monoclonal Antibodies.** The primary rat anti-mouse antibodies used for immunofluorescence staining included M5114 specific for class II MHC I-A molecules (40), YNI.1 specific for intercellular adhesion molecule 1 (ICAM-1) (41), and 7D4 specific for the IL-2 receptor α chain (42), all were obtained from the American Type Culture Collection. In addition, MK-2 specific for vascular cell adhesion molecule 1 (VCAM-1) (41) was obtained from Dr. Paul Kincade (University of Oklahoma, Tulsa, OK). Purified anti-mouse CD28 (Pharmingen, San Diego, CA) was used in some antigen presentation assays.

**RNA Isolation and Reverse Transcription PCR Analysis of IL-2 Gene Expression.** A.E7 cells were cocultured on monolayers of IFN-γ-treated SVEC4-10 cells in 2 ml polystyrene wells at 10^6 T cells per well in the absence or presence of 5 x 10^6 BALB/c spleen cells plus or minus 100 μg/ml PCC. Viable T cells were harvested from the monolayers after 6 h, total cellular RNA was isolated using RNaZol™ (Biotexc Laboratories, Inc., Houston, TX), and reverse transcriptase (RT)-mediated synthesis of cDNA was performed with an oligo(dT) primer. The cDNA samples were precipitated using 0.2 M ammonium acetate (pH 4.5) and 2 vol absolute ethanol. The cDNA samples were then resuspended in 50 μl of water, and 2.5 μl was used for PCR amplification with mouse IL-2 (43) and β-actin–specific primers. Following amplification, the PCR products were analyzed by ethidium bromide staining in agarose gels using standard techniques (44).

**Results**

**Phenotype of SVEC4-10 Cells.** Effective antigen-presenting activity to CD4⁺ T cells depends on class II MHC expression by the APC. SVEC4-10 cells do not constitutively express class II MHC, but they can be induced to do so by treatment with IFN-γ (Fig. 1), as has been reported (13). Expression by SVEC4-10 cells of ligands for lymphocyte integrins was previously uncharacterized. The SVEC4-10 line constitutively expresses VCAM-1, but not ICAM-1 (Fig. 1), and these phenotypic features were not altered by IFN-γ treatment (not shown).

**SVEC4-10 Endothelial Cell Antigen Presentation to T Cell Clones.** The capacity of the SVEC4-10 line to support antigen-specific stimulation of murine helper T cell clones was tested using a Th2-type murine helper T cell clone, D10.G4, and two Th1 type clones, A.E7 and AR100. Antigen recognition by all three clones is restricted to class II MHC alleles of the k haplotype; the SV4EC-10 cell line was derived from an H-2k mouse (C3H/HeJ). IFN-γ-treated SV4EC-10 cells were effective APCs for the Th2-type T cell clone D10.G4, and two Th1 type clones, A.E7 and AR100. Antigen recognition by all three clones is restricted to class II MHC alleles of the k haplotype; the SV4EC-10 cell line was derived from an H-2k mouse (C3H/HeJ). IFN-γ-treated SV4EC-10 cells were effective APCs for the Th2-type T cell clone D10.G4, and two Th1 type clones, A.E7 and AR100, as assessed by antigen-specific induction of T cell proliferation. Endothelial cells not treated with IFN-γ did not present antigen. In contrast, two H-2k-restricted Th1-type T clones, A.E7 and AR100, were not stimulated to proliferate by the IFN-γ–treated endothelial cell line (Fig. 2).

**Allogeneic Spleen Cells Complement SVEC4-10 Antigen Presentation to Induce A.E7 T Cell Proliferation.** Antigen-induced autocrine growth factor production by Th1 clones is generally more dependent on accessory cell costimulator molecules than are Th2 clones. Therefore, the inability of SVEC4-10 cells to stimulate Th1 cell proliferation, shown in Fig. 2, may have been due to a lack of costimulators. Allogeneic spleen cells have been reported to provide costimulatory activity for A.E7 activation in experimental systems using chemically modified APCs (28). To test whether allogeneic spleen cells would complement SVEC4-10 antigen presentation and allow
a proliferative response to occur, irradiated BALB/c spleen cells were added to the cocultures of the endothelial and A.E7 cells. Under these conditions, antigen-induced proliferation of the T cells was observed (Fig. 3). These spleen cells did not induce antigen-specific proliferation of A.E7 cells in the absence of the SVEC4-10 cells (not shown), consistent with their inability to express the I-E\(^k\) class II MHC molecule which restricts A.E7 antigen recognition. Furthermore, the observed proliferation of the T cells was not due to alloreactivity because it was not observed in the absence of the specific antigen.

**SVEC4-10 Cell Presentation of Preformed PCC Peptide Does Not Induce A.E7 T Cell Proliferation.** The failure of the SVEC4-10 cells to support antigen-induced proliferation of A.E7 cells might be observed if the endothelial cell line was incapable of processing and generating the specific peptide recognized by A.E7 cells. The general capacity of the SVEC4-10 cells to internalize, process, and present soluble protein antigens to class II-restricted T cells was demonstrated in experiments with the D10.G4 clone (Fig. 1). Nonetheless, there may have been a selective ability of the SVEC4-10 cells to process conalbumin but not PCC. Allogeneic spleen cells could theoretically have generated the appropriate peptides that were then subsequently delivered to the endothelial cells for presentation to the T cells. The data in Fig. 4 indicate that this is not the case. Addition of immunoreactive PCC peptide 89-104 to cocultures of SVEC4-10 and A.E7 cells did not result in T cell proliferation, as was the case for intact PCC, unless allogeneic spleen cells were added. It is therefore likely that the spleen cell effect of permitting A.E7 proliferation was due to the provision of costimulatory molecules.

**Allogeneic Spleen Cells Complement SVEC4-10 Antigen Presentation to Induce IL-2 Gene Expression.** One demonstrated role of costimulators in T cell proliferation is to provide signals required for efficient IL-2 gene transcription (23). A failure of SVEC4-10-mediated induction of proliferation of A.E7 cells could be the consequence of the failure of these endothelial cells to stimulate T cell expression of the IL-2 gene. To test this hypothesis, we measured IL-2 in supernatants from cocul-
tured with BALB/c cells plus antigen, in the absence of SVEC4-10 cells (not shown). No IL-2 gene-specific amplification product was seen when A.E7 cells were eDNA is 450 bp; each increment in the control DNA ladder is 100 bp.

Figure 5. SVEC4-10 cells lack costimulators for A.E7 IL-2 gene expression. IL-2 gene expression in A.E7 cells was determined by RT PCR analysis of RNA isolated from the T cells after 6 h of coculture with IFN-γ-treated SVEC4-10 cells plus antigen even when anti-mouse CD28 mAb was added at 25 or 100 μg/ml (data not shown).

Table 5. SVEC4-10 cell antigen presentation to A.E7 cells upregulates responsiveness to exogenous IL-2. A.E7 cells were cocultured with IFN-γ-treated SVEC4-10 cells plus antigen for 18 h, varying concentrations of recombinant murine IL-2 were added to triplicate microwells, and the cultures were incubated for an additional 18 h. T cell proliferation was determined by measuring [3H]thymidine incorporation during the final 6 h of culture. The results of one experiment are shown; two additional experiments had similar results.
by SVEC4-10 cells also did not induce anergy in A.E7 cells. As a positive control for anergy induction, the proliferative responses of A.E7 cells to PCC and splenic APCs was measured 7 d after exposure to ECDI-treated spleen cells plus PCC peptide. This response was 75% inhibited (Fig. 8 B) compared to control cells cultured with ECDI-treated spleen cells but no peptide. The response to IL-2 at this 7 d time point was not altered by previous exposure to antigen, using either ECDI-treated spleen or SVEC4-10 cells.

Discussion

The present experiments were aimed at defining the functional interactions between endothelial cells and T lymphocytes using an in vitro mouse model system. In particular, the antigen presentation capabilities and costimulatory activities of a well-differentiated mouse endothelial cell line, SVEC4-10 were examined. The SVEC4-10 cell line was derived from a primary culture of mouse lymph node vascular endothelium by SV40 virus immortalization (13). In addition to the previously reported markers of endothelial cell differentiation, such as acetylated-LDL receptor and factor VIII expression, we have shown it constitutively expresses VCAM-1 and not ICAM-1. VCAM-1 is a ligand for VLA-4 and is potentially important for trafficking of antigen-specific T cells to sites of antigen challenge. In addition, VCAM-1 may deliver costimulatory signals to CD4+ T cells (46, 47).

The absence of ICAM-1 expression by this cell line should permit analysis of VCAM-1-dependent adhesive and stimulatory interactions in isolation from ICAM-1 effects.

Figure 7. SVEC4-10 cell antigen presentation to A.E7 cells upregulates expression of the IL-2R B chain. A.E7 cells were cocultured with SVEC4-10 cells with or without PCC antigen in 2-ml culture wells for 18 h. Viable T cells were then harvested, stained for expression of the p55 subunit of IL-2R using the 7D4 antibody, and analyzed by flow cytometry. Dotted tracings indicate fluorescence histograms of cells incubated using a primary rat anti-CD8 antibody as a control.
The ability of SVEC4-10 cells to support antigen-specific stimulation of the proliferation of the Th2 helper T cell clone D10.G4 indicates that this endothelial cell line can take up, process, and present a soluble protein antigen in a class II MHC-restricted manner. Although full activation of Th2 cells has less stringent requirements for costimulators, it is possible that IFN-γ treatment of the endothelial cell line, required for class II MHC expression, also induced the expression of unidentified molecules required for Th2 cell proliferation. In contrast, SVEC4-10 cells were not capable of stimulating the proliferation of two different Th1 T cell clones, including the well-described A.E7 clone. This failure to induce proliferation correlated with the failure of the SVEC4-10 cells to induce the appearance of IL-2 mRNA. The data indicate that these endothelial cells lack costimulatory molecules required by A.E7 cells for antigen-induced activation of autocrine IL-2-mediated growth. The required costimulatory activity can be provided by third party allogeneic spleen cells. The ability of SVEC4-10 endothelial cells to stimulate proliferation of Th2 but not Th1 cells is consistent with a recent report that antigen presentation by brain microvessel endothelium also leads to Th2 but not Th1 cell autocrine growth factor secretion and proliferation (48).

The best-defined costimulatory system in T cell activation is that involving B7-CD28 interactions (23, 49-55). There are two reasons why it is unlikely that the absence or presence of B7 is playing a significant role in the T cell functional responses described here. First, there is evidence that B7 must be present on the APC for it to optimally costimulate T cell proliferation (56, 57). In contrast, costimulatory activity for A.E7 proliferation that is missing on the SVEC4-10 cells can be provided by nonantigen-presenting, third party allogeneic spleen cells. Second, anti-CD28 antibody used under conditions reported to costimulate certain other T cells in culture (58) did not have any effect on the ability of the SVEC4-10 cells to induce proliferation of the A.E7 cells. In addition to B7, both VCAM-1 and ICAM-1 have been implicated as costimulators of Th cell IL-2 production and proliferation (46, 47, 59-61). ICAM-1 is not expressed on the SVEC4-10 cells under the conditions used in the experiments reported here, and this is a possible reason for the incomplete T cell activation observed in response to the antigen presentation by the endothelial cell line. We believe that this is unlikely for three reasons. First, previous reports indicate that VCAM-1 and ICAM-1 are costimulators of resting T cells but not necessarily of T cells previously activated by antigens (47, 59-61). In fact, it has been shown that IL-2 production by T cells previously activated by superantigen was not costimulated by either VCAM-1 or ICAM-1 (47). In the studies reported here, the T cell clones were activated by antigen within 14 d of the experimental culture. Second, when directly compared, VCAM-1 and ICAM-1 each independently costimulated TCR-mediated proliferation of naive T cells equally well; that is, the presence of both ligands was not necessary for optimal T cell activation (47). Because the SVEC4-10 line expresses VCAM-1, there is no reason to assume that ICAM-1 is also needed. Third, some ICAM-1 expression can be induced on the SVEC4-10 line by TNF treatment, but TNF did not render the endothelial line competent at stimulating a proliferative response of A.E7 cells (data not shown).

Despite the lack of costimulatory activity for Th1 cell proliferation, SVEC4-10 antigen presentation to A.E7 cells did not induce anergy. The consequences of antigen presentation by viable but costimulator-deficient APCs to Th1 cells is largely uncharacterized. Most studies of clonal anergy, and in particular of anergy induced in the A.E7 clone, have been performed using nonviable, chemically fixed APCs. Although some "nonprofessional" APCs may induce T cell anergy (29, 30), the consequences of antigen presentation by many other cell types have not been carefully examined. The present results indicate that induction of anergy does not strictly correlate with the lack of costimulators for antigen-induced Th1 proliferative responses. Because the molecular events involved in costimulation of IL-2 production (62) and anergy induction (37, 63) are, at best, incompletely defined, there is no a priori reason to assume that the two results of costimulator-deficient antigen presentation are necessarily linked. In fact, there is evidence that blockade of the B7-CD28 costimulatory pathway in vivo can inhibit T cell-dependent immune response to protein antigens without induction of tolerance to those antigens (64). The experimental system described here may help to distinguish divergent mechanisms underlying costimulation of IL-2 production versus regulation of anergy induction.

The partial activation response of A.E7 to SVEC4-10 antigen presentation, characterized by induction of IL-2 receptor expression suggests a possible physiological role for endothelial antigen presentation. Antigen-specific interactions of T cells with endothelium in the lumena of microvasculature could upregulate surface molecules required for tight adhesion and extravasation of T cells into tissues without initiating proliferative responses. This would result in selective enrichment of antigen-specific T cells at a site of antigen exposure. Furthermore, upregulation of IL-2R and possibly other molecules by endothelial antigen presentation might result in enhanced functional responses of these T cells to subsequent antigen exposure in extravascular sites. In other words, endothelial cells may direct antigen-specific T cells to sites of extravascular antigen and prepare T cells to respond to tissue-based APCs.

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