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The effect of allo-injury in an in vitro model of allograft microvasculature

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Abstract Endothelial cells are critical to the integrity of allograft vasculature and can be damaged by alloreactive T cells or soluble mediators of alloreactivity. The biochemical effects of T cell-mediated damage to the endothelial cells have been studied, but not the structural and morphological effects of allo-injury on endothelial cells in the allograft. We utilized an assay that reproduces microvasculature in vitro to study the effect of alloreactivity on endothelial cells. In this assay, endothelial cells are induced into capillary-like networks that simulate microvascular capillaries. We studied the effect of allogeneic T cells and of soluble mediators from both mixed lymphocyte cultures (MLCs) and rejected heart allograft tissue on the in vitro capillaries. We found that both allogeneic T cells and soluble mediators inhibit the formation of the in vitro endothelial capillaries, suggesting that they cause a mild-to-moderate dysfunction of the endothelial cells. The inhibitory effect of the soluble mediators seems to be mediated, at least partly, by IFN-γ, since this effect was prevented by antibody to IFN-γ. Furthermore, pre-incubation of the in vitro capillaries with IFN-γ appeared to magnify the effect of allogeneic T cells, as shown by a complete breakdown of well-formed in vitro capillary networks. Our experiments suggest that the in vitro capillary-tube model reflects structural injury to allograft vasculature by alloreactive T cells and their soluble mediators.

Keywords Endothelial cells · Matrigel · Alloreactive T cells · IFN-gamma · SVEC 4-10

Introduction

Endothelial injury in acute rejection episodes seems to correlate with the severity of rejection and with long-term graft survival [1]. The histopathological features of endothelial injury include inflammation of the endothelium, detachment of endothelial cells (ECs) from the underlying matrix, and disruption of the cell-to-cell contacts [2, 3, 4, 5, 6]. All these lead to the loss of tubular morphology of the endothelium. These morphological features indicate that allogeneic T cells and soluble mediators from these T cells cause structural damage to microvasculature endothelium in allografts.

Endothelial injury in allografts has been studied mainly by its morphological features on histology. Transmission and scanning electron microscopic studies on coronary artery specimens from heart allografts with severe rejection have shown that lymphocytes, macrophages, and smooth muscle cells invade the intima, with the lymphocytes being in close proximity to the ECs. Endothelial injury in these specimens is also characterized by vacuolization of the ECs and partial denudation of the endothelium from the underlying matrix. The endothelium in these specimens show gaps between the ECs, within which leukocytes and platelets are often found [5]. These findings show

that the tubular structure of endothelium is disrupted by allo-injury.

Host mechanisms causing endothelial injury have been investigated. Pober et al. have analyzed the interactions between ECs and alloreactive T cells in vitro using EC monolayers [7]. These studies indicate that ECs can present antigens, although not as efficiently as professional antigen-presenting cells [8]. ECs are also good targets for cytotoxic, but not naive, T cells [9]. Although these studies have provided valuable information on the interaction between ECs and T cells, most of them have focused on T cells and other effector mechanisms. Very few studies have focused on the structural consequences of these T cell-mediated injuries in the endothelial cells.

We have described an in vitro model of endothelial injury that utilizes endothelial cells in a capillary-tube configuration rather than monolayers [10]. This capillary-tube assay (CTA) reflects more accurately the structural architecture of capillaries in vivo than ECs in monolayers. On the other hand, the CTA, in comparison to in vivo models, provides a facile system for one to investigate the molecular mechanisms of endothelial injury. In this study, we used the CTA as an in vitro model of allograft microvasculature to study the consequences of allo-injury to the structure of capillaries. Our primary objective was to determine whether the endothelial injury in allografts could be reproduced in vitro with the capillary-tube assay. Our second objective was to determine if either the cellular or the soluble mediators of cellular injury, or both, cause endothelial injury in the capillary-tube assay.

Material and methods

Transformed mouse endothelial cell line SVEC 4-10 [11] was kindly provided by Dr. K. O'Connell, F.D.A., Washington DC. Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen (Carlsbad, Calif., USA). Matrigel, IFN-γ, IL-2, anti IFN-γ (clone XMG1.2), anti-IL-2 (clone S4B6) monoclonal antibodies, anti-mouse CD 106 [vascular cell adhesion molecule-1 (VCAM-1), clone 429], and FITC-conjugated polyclonal antibody were obtained from BD Biosciences (Bedford, Mass., USA).

An in vitro model to study endothelial injury

To study endothelial injury, we used a model in which endothelial cells (SVEC 4-10) are induced to form capillary tube-like networks on a laminin-rich matrix (Matrigel). Matrigel (50 µl per well) was dispensed at 4°C to coat 96-well tissue culture plates and allowed to polymerize at 37°C for at least 12 h. Murine SVEC 4-10 endothelial cells were dispersed by trypsinization, counted, and then suspended in DMEM supplement with 10% fetal bovine serum. SVEC 4-10 cells, at a density of $1–4\times10^3$ cells per well in a final volume of 150 µl per well, were plated on Matrigel and then cultured in 5% CO_2 at 37°C.

Incubation of cytokines and antibodies to cytokines with endothelial capillaries

To determine the effect of inflammatory cytokines on endothelial capillaries, we incubated IFN-γ (1–400 pg/ml) with SVEC 4-10 cells on Matrigel at the onset of culture or after capillary tubes had matured (24 h after culture on Matrigel).

In independent experiments, the anti IFN-γ (clone XMG1.2) or anti IL-2 (clone S4B6) monoclonal antibody was incubated with SVEC 4-10 cells 1 h before mixed lymphocyte culture (MLC) supernatant was added to the culture.

Incubation of MLC supernatant with endothelial capillaries

To determine the effect of in vitro alloreactivity on endothelial capillaries, we utilized supernatants from mixed lymphocyte culture. Allogeneic mixed lymphocyte cultures were done between splenocytes from Balb/c ($H\text{-}2^d$) and C57Bl/6 ($H\text{-}2^k$) mice. Spleens from stimulator mice (Balb/c) were minced, and lymphocytes were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation. Responder lymphocytes were obtained similarly from spleens of C57Bl/6 mice. Proliferation of responder cells (C57Bl/6 mice) to an equal amount (2×10^5) of 50-Gy gamma-irradiated stimulator cells (Balb/c mice) was tested in a standard one-way MLC assay. Cell mixtures were cultured in 96-well round-bottomed microtiter plates (Corning–Costar) for 5 days. Isogeneic MLCs were done between C57Bl/6 mice (responder) and irradiated C57Bl/6 (stimulator) lymphocytes in the same manner. The supernatants from these cell mixtures were incubated with SVEC 4-10 cells on Matrigel at the onset of culture or after capillary tubes had matured (24 h after culture on Matrigel).

Measurement of cytokine concentrations in MLCs

The level of IFN-γ and IL-2 in the supernatant from the MLCs was analyzed by two-antibody capture ELISA. We coated 96-well flat-bottomed plates with 50 µl of the

appropriated unconjugated capture Ab at 2 μg/ml in 0.1 mol/l $NaHCO_3$ (pH 8.2) overnight at 4°C. Plates were washed twice with 0.05% Tween-20 in phosphate buffer solution (PBS) and blocked with 200 μl of 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Plates were washed twice, and supernatants from the MLCs were added and incubated overnight at 4°C. Plates were washed four times, incubated with 100 μl of the appropriate biotinylated detecting Ab (1 μg/ml) for 45 min at room temperature, and washed six times, and then 100 μl of 1/1,000-diluted avidin–peroxidase was added to each well and incubated for 30 min at room temperature. Plates were washed eight times, and 100 μl of freshly prepared 2,2′-azino-bis-[3-ethylbenziazoline-6-sulfonic acid] (ABTS) substrate (Kirkegarrd & Perry, Gaithersburg, Md., USA) was added to each well. The reactions were stopped at 5 min by addition of 100 μl of 1% sodium dodecyl sulfate (SDS) (Sigma). Plate optical density (OD) values were measured at 405 nm on a Biotek EL311s automated microplate reader. We determined cytokine concentrations by comparing them to a standard curve generated with recombinant cytokines, using the Biotek EL311 best curve-fit software. The lower limit of detection for the cytokines is 0.5 U/ml for IL-2 and 1.0 U/ml for IFN-γ.

Incubation of heart allograft and isograft extracts with endothelial capillaries

Heterotrophic non-vascularized heart transplantation was performed between C57Bl/6 and Balb/c mice as described previously [12]. Briefly, hearts from C57Bl/6 newborn mice were transplanted to the ear pinna of Balb/c recipients. The recipient mice were anesthetized by inhalation of halothane, oxygen and nitrous oxide. A pocket on the dorsum of one ear was created between the skin and cartilage plate toward the distal edge of the ear. The hearts of the newborn mice were procured and transplanted to the ear pinna of recipient mice. Donor tissue was eased into the base of the pocket near the distal edge of the ear. Healing of the pocket incision occurred after a short period of inflammation, and, usually, within 5 to 6 days, pulsatile activity of the transplanted hearts was detected by EKG tracing. Electrocardiogram monitoring was done on the 5th day after transplantation and every 2 days so that we could confirm graft function. At day 7 after transplantation, grafts were harvested, homogenized, and extracted in DMEM at 4°C. Allograft and isograft extracts were incubated with SVEC 4-10 cells on Matrigel at the onset of culture or after capillary tubes had matured (24 h after culture on Matrigel). The concentration of IFN-γ in these extracts was measured by two-antibody capture ELISA as described above.

Generation of alloreactive T cells and co-culture with endothelial cells

C57Bl/6 ($H\text{-}2^b$) mice were immunized with C3H ($H\text{-}2^k$)-origin SVEC 4-10 cells, 10^7 cells in 500 μl of Freund's adjuvant. Ten days after immunization, we obtained enriched T cells from spleens of immunized C57Bl/6 mice by passing the splenocytes through a nylon-wool column. These T cells were incubated with SVEC 4-10 cells with or without Concanavalin A (Con A; 2 μg/ml) or IL-2 (10 pg/ml) for 24 h. The SVEC 4-10 cells were trypsinized, and the mixture of the SVEC 4-10 and T cells was cultured on Matrigel.

In another set of experiments, alloreactive T cells were activated with Con A or IL-2 and cultured in the absence of SVEC 4-10 cells for 24 h. Subsequently, these activated alloreactive T cells were added to SVEC 4-10 cells at the onset of culture on Matrigel or after the capillary tubes had matured (24 h after culture on Matrigel).

In the final set of experiments with alloreactive T cells, SVEC 4-10 cells were first incubated for 24 h on Matrigel to form mature capillary tubes. These capillary tubes were then incubated with IFN-γ for 6 h and then with activated alloreactive T cells for another 24 h. Control capillary tubes were incubated with only IFN-γ for 24 h.

Assay for endothelial cell activation and cell viability

Vascular cell adhesion molecule-1 (VCAM-1) expression on SVEC 4-10 cells was determined 24 h after incubation with activated alloreactive T cells by immunofluorescence staining, according to the manufacturer's instructions, in monolayers and in the capillary-tube assay. Briefly, the cultures were fixed with 3.7% formaldehyde solution at 37°C then incubated with primary antibody (CD 106, VCAM-1). After being washed, the cultures were then incubated with secondary antibody, FITC-conjugated polyclonal antibody, and examined under an inverted fluorescence microscope.

Viability of the SVEC 4-10 cells was determined after 24 h incubation with activated alloreactive T cell in monolayers and in the capillary-tube assay by trypan blue (Sigma) exclusion. Briefly, trypan blue (1%) was diluted 3:7 with PBS and filtered immediately before being used. The diluted stain (100 μl) was added to the cell suspension, 150 μg/ml, and then incubated at room temperature for 2 to 3 min before microscopic examination with a hematocytometer. Cells stained with trypan blue were categorized as non-viable.

The *Principles of Laboratory Animal Care* was followed and the Committee for the Humane Use of Animals at SUNY Upstate Medical University, Syracuse, New York, approved our protocol.

Statistical analysis

All experiments were done in triplicate and repeated twice. Three parameters of endothelialitis were studied in this model: inhibition of capillary-tube formation, reduction of capillary-tube networks, and disruption of mature capillary tubes. For the reduction of capillary-tube networks, the number of intersections of capillary tubes per well was counted under an inverted microscope (Nikon Eclipse TS 100). Mean ± SD of the number of intersections of capillary tubes per well in the control and experimental groups were analyzed by ANOVA. We made a Bonferroni adjustment to the type I error in order to preserve the overall α level at 0.05.

Results

Time cause and morphological parameters of the in vitro microvascular capillaries

Endothelial cells cultured on Matrigel stopped proliferating and started forming capillary-like networks within 4 to 6 h. These networks matured within 16 to 24 h, after which the in vitro capillaries were resistant to breakdown for another 24 to 48 h. At the end of 72 h the in vitro capillary networks spontaneously broke down, leaving individual endothelial cells that eventually died within the next 48 h.

In this assay, we measured three morphological parameters of in vitro endothelial dysfunction: inhibition of capillary-tube formation, reduction of capillary-tube networks, and disruption of mature endothelial capillaries. The first two parameters were observed at 24 h after we had plated SVEC 4-10 cells on Matrigel. The last parameter was studied after capillary tubes had matured (24 h after culture) and was observed at 48 h after the initial endothelial cell seeding. Mild and moderate endothelial dysfunction was manifested by reduction and inhibition of capillary-tube networks while severe endothelial dysfunction was manifested by disruption of mature endothelial capillaries.

Effect of alloreactive T cells and activated alloreactive T cell on endothelial capillaries

The SVEC 4-10 endothelial cells were incubated with alloreactive T cells under different conditions so that we could determine the effect of the T cells on endothelial cell function. First, alloreactive T cells were co-cultured with SVEC 4-10 cell monolayers for 24 h, and both SVEC 4-10 and the alloreactive T cells were plated on Matrigel. Under these conditions, in the presence of alloreactive T cells, the SVEC 4-10 cells formed capillary networks, but the number of networks was significantly less than in the control SVEC 4-10 cells (27.67 ± 2.5 in the control group vs 4.7 ± 0.6 in the experimental group, $P < 0.01$; Table 1; Fig. 1).

Table 1 Effect of alloreactive T cells and supernatant from mixed lymphocyte culture on endothelial dysfunction

Parameter	Number of intersection of capillary tubes
Control	27.67 ± 2.5
Pre-incubated SVEC 4-10 cells with alloreactive T cells	4.7 ± 0.6*
Pre-incubated SVEC 4–10 cells with alloreactive T cells in media containing Con A or IL-2	0
No pre-incubation/addition of Con A-activated alloreactive T cells at the onset of culture	2.3 ± 1.5*
No pre-incubation/addition of IL 2-activated alloreactive T cells at the onset of culture	2.0 ± 1.0*
Supernatant from MLC	0
Supernatant from MLC + anti-IFN-γ antibody	15.00 ± 3.00

* $P < 0.05$ with ANOVA followed by Bonferroni test

Second, alloreactive T cells were co-cultured with SVEC 4-10 cells for 24 h but, this time, in the presence of Con A (2 μg/ml) or IL-2 (10 pg/ml). In the presence of these activated alloreactive T cells, the SVEC 4-10 cells appeared more dysfunctional because they did not form capillary tubes at all. Indeed, in the presence of activated alloreactive T cells, the SVEC 4-10 cells were found in clumps on Matrigel rather than in capillary networks (Fig. 2).

Third, alloreactive T cells were first incubated with Con A (2 μg/ml) or IL-2 (10 pg/ml) for 24 h in the absence of SVEC 4-10 cells. These activated alloreactive

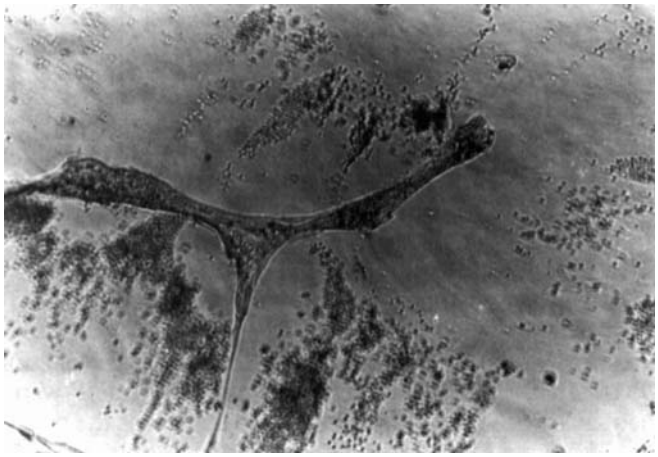

Fig. 1 Effect of alloreactive T cells on capillary-tube networks. A significant reduction in the number of capillary-tube networks was observed in the presence of alloreactive T cells

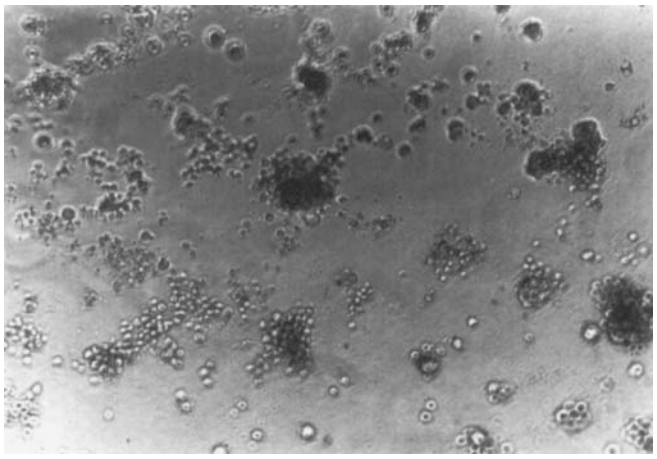

Fig. 2 Effect of activated alloreactive T cells on capillary-tube formation. Activated alloreactive T cells completely inhibited capillary-tube formation

T cells were then added to the SVEC 4-10 cells at the onset of culture on Matrigel. Under this condition the SVEC 4-10 cells formed capillary networks, but the number of networks, again, as in the first condition, was reduced significantly (27.67 ± 2.5 in the control group vs 2.3 ± 1.5 and 2.0 ± 1.0 in the Con A and IL-2 activated group, respectively, $P < 0.01$; Table 1). In the control experiments, neither IL-2 nor Con A had an effect on capillary-tube formation.

Activated alloreactive T cells were incubated with mature endothelial capillaries that had been pre-incubated with IFN-γ. Under these conditions, the mature endothelial capillaries (24 h after initial culture on Matrigel incubated for 6 h with IFN-γ 400 pg/ml) were disrupted when further incubated with alloreactive T cells. Incubation of the endothelial capillaries with IFN-γ alone or with alloreactive T cells alone did not have any effect on mature capillary networks. Thus, mature capillary tubes were susceptible to injury by alloreactive T cells only when the endothelial cells in the capillaries were pre-incubated with IFN-γ.

Taken together, the experiments with the alloreactive T cells suggested that the capillary tubes were most susceptible to injury by activated allogeneic T cells, especially when the endothelial cells were treated with IFN-γ, whereas the capillary tubes were least susceptible to injury by allogeneic T cells alone.

Effect of activated alloreactive T cell on endothelial activation and cell viability

We studied the expression of VCAM-1 on endothelial cells as a marker of endothelial cell activation. We found that after 24 h of incubation with activated alloreactive T cells, a majority of the SVEC 4-10 cells in monolayers as well as in capillary networks were stained positively

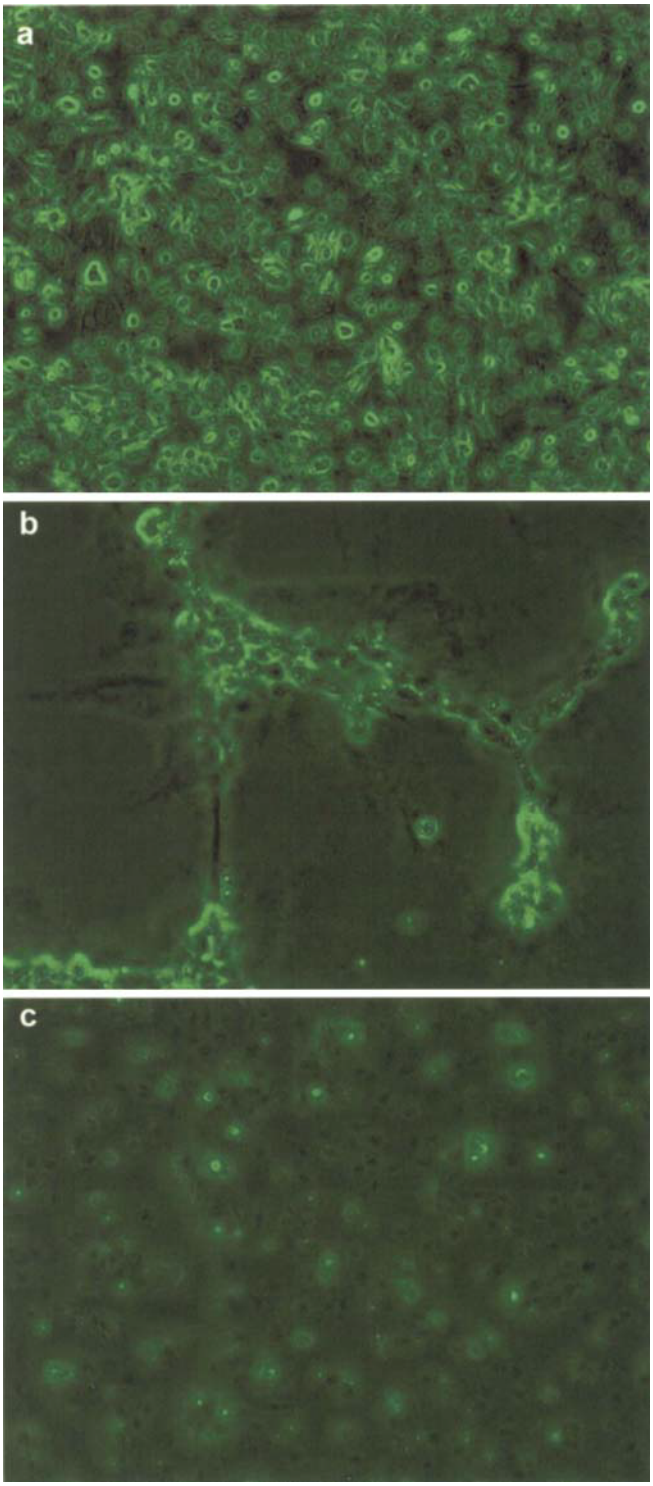

Fig. 3a-c SVEC 4-10 cells incubated with activated allogeneic T cells and stained antibody to VCAM-1 in monolayers (**a**) and in capillary networks (**b**). **c** SVEC 4-10 cells in monolayer (control) stained with antibody to VCAM-1. Similar staining pattern was observed with SVEC 4-10 cells in capillary networks not incubated with activated alloreactive T cells (data not shown)

for VCAM-1 (Fig. 3a, b). Control SVEC 4-10 cells in monolayers or in capillary networks were not stained positively for VCAM-1 (Fig. 3c).

The viability of SVEC 4-10 cells after 24 h incubation with activated alloreactive T cells in monolayers and capillary-tube assay was greater than 95% by trypan blue exclusion assay.

Effect of MLC supernatant on endothelial capillaries

To determine if the endothelial injury in this in vitro model was induced by lymphocyte-derived soluble mediators, we tested the effects of MLC supernatants on capillary-tube formation. Supernatants from allogeneic MLCs performed between C57/Bl6 mice and irradiated Balb/c lymphocytes were added to the capillary-tube assay. We found that supernatants from the allogeneic MLCs inhibited capillary-tube formation (Fig. 4). Supernatants from isogeneic MLCs had no effect on capillary-tube formation.

Effect of rejecting heart allograft extracts on endothelial capillaries

Heart allografts exchanged between C57Bl/6 mice donors and Balb/c mice recipients underwent acute rejection, with a mean survival time of 7 days. The rejection was characterized by a loss of cardiac impulse on EKG and severe endothelial and myocardial injury on histological examination. Isografts continued to have vigorous cardiac impulses and did not show any injury on histology. To determine the effect of soluble mediators from rejecting allografts, we incubated extracts from allografts or from isografts with murine endothelial cells (SVEC 4-10) on Matrigel, either before capillary-tube formation or after the capillary tubes had matured. When added to the endothelial cells before tube formation, extracts from rejecting allografts (Fig. 5a) but not from isografts (Fig. 5b) inhibited capillary-tube formation. However, when the extracts were incubated with the SVEC 4-10 cells after formation of stable capillary tubes, neither extract had any effect.

Role of IFN-γ in endothelial dysfunction caused by MLC supernatants and allograft extracts

Since IFN-γ is a key mediator of endothelial injury in allografts, we tested the effect of IFN-γ alone on capillary-tube formation by SVEC 4-10 cells. As reported previously by us [11] and others [13], IFN-γ 10–400 pg/ml inhibits capillary-tube formation. To determine if IFN-γ in the MLC supernatants and allograft extracts played a role in the endothelial dysfunction, we deter-

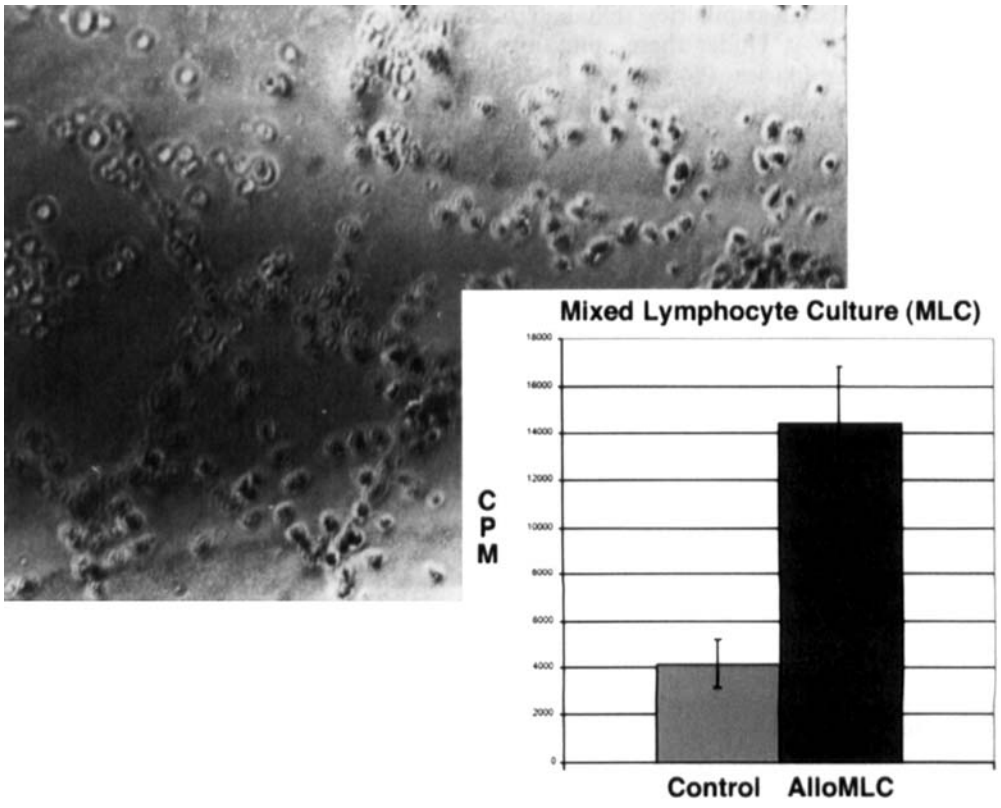

Fig. 4 Effect of supernatants from MLCs on capillary-tube formation. Similar to allograft extracts, supernatants from alloreactive lymphocytes in MLCs inhibited in vitro capillary-tube formation. Supernatants from non-reactive lymphocytes had no affect on this parameter of endothelial dysfunction (data not shown). Insert demonstrates the degree of alloreactivity in MLCs as measured by ^{3}[H]-thymidine incorporation into proliferating lymphocytes and as expressed as counts per minute (*CPM*)

mined the concentrations of IFN-γ in the supernatant and allograft extracts and found them to be 204.1 ± 2.8 pg/ml and 160.53 ± 31.84 pg/ml, respectively. Next, we pre-incubated the endothelial cells (SVEC 4-10) with an IFN-γ blocking antibody 1 h before adding the MLC supernatants. The addition of IFN-γ antibody reduced the effects of the allogeneic MLC supernatants, allowing the capillary tubes to form, albeit with a decreased number of capillary-tube intersections (Table 1).

Discussion

The lack of an in vitro model to study the effect of allo-injury on endothelial cells in allografts prompted us to adapt an endothelial capillary tube-formation assay to mimic, in vitro, the morphological features of endothelial injury in allografts. We found that endothelial injury that led to loss of capillary morphology, similar to that in allografts, could be reproduced in this assay with soluble extracts from rejecting cardiac allografts. We also found that both cellular and soluble mediators of allo-injury can elicit a similar endothelial injury in the capillary-tube assay. Activated alloreactive T cells and MLC supernatants caused complete inhibition of capillary-tube formation. Finally, we showed that IFN-γ, a known mediator of endothelial injury in endothelialitis, was a key cytokine in the inhibition of capillary-tube formation by the soluble mediators from MLC and that this cytokine increased the susceptibility of the endothelial capillaries to allo-injury by T cells. All these data support the notion that the capillary-tube assay is a novel in vitro model for the study of endothelial injury in allografts.

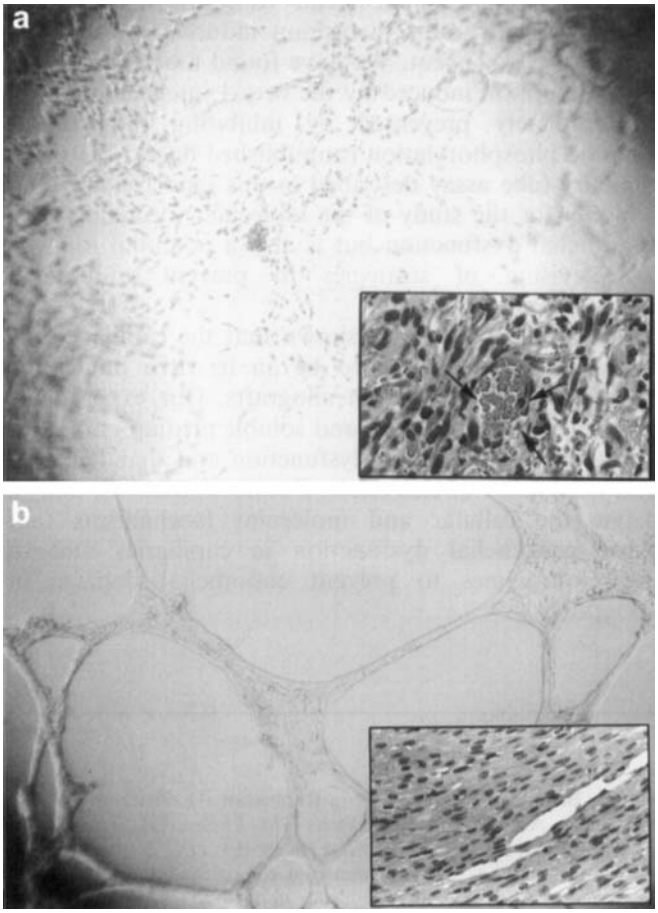

Fig. 5a, b Effect of soluble extracts from non-vascularized cardiac isografts and allografts. **a** Soluble extracts from rejecting heart allografts (non-vascularized) inhibited in vitro mouse capillary-tube formation on Matrigel. **b** In contrast, extracts from isografts had no effect. Inserts in **a** and **b** show histological sections (×20) of allografts and isografts stained with H&E that demonstrate severe rejection and no rejection, respectively

There are several biochemical assays of endothelial function but none of these measures the fundamental morphological property of endothelial cells to form capillary tubes. A commonly used biochemical assay measures nitric oxide (NO) production by endothelial cells [14, 15, 16]. This assay has been used in transplant recipients to determine the effect of calcineurin inhibitors on NO production in vivo [17]. The NO production assay provides valuable information on the vasomotor regulation of the vasculature by the calcineurin inhibitors but does not provide insights into endothelial dysfunction in allografts. Other biochemical assays that are less commonly used in transplantation measure the release of prostacyclin and thrombomodulin by endothelial cells [18, 19]. Both these are sensitive biochemical indicators of endothelial injury. We have studied prostacyclin release in the capillary-tube assay in response to injury and it is, indeed, possible for one to discern the effects of various injuries on prostacyclin release by the endothelial capillaries in the capillary-tube assay (unpublished data). The capillary-tube assay by itself provides valuable morphological information relevant to endothelial dysfunction, and, by combining it with a sensitive biochemical assay, one could extend the discriminatory capacity of the capillary-tube assay.

Discrete and objectively measurable injury from both cellular and soluble mediators of alloreactivity is caused by morphological changes in the capillary-tube assay. With regard to the latter, it is notable that the concentration of IFN-γ in the MLC supernatants and in rejecting allografts was similar to that at which IFN-γ alone caused inhibition of capillary tubes. This observation by itself does not support a casual relationship between IFN-γ and endothelial injury caused by the MLC supernatants in the capillary-tube assay. However, two of our other observations suggest that IFN-γ is an important mediator in allografts. First, anti-IFN-γ antibody partially prevented endothelial injury from the

MLC supernatants. Second, the incubation of IFN-γ with the capillary tubes significantly enhanced the injury to the capillary networks by T cells. Our observation is also consistent with other studies that have shown that IFN-γ is injurious to the endothelial cells [20] in allograft and other tissue injury. Finally, these experiments with IFN-γ suggest a strategy that would further characterize other cytokines that can potentially cause endothelial injury in allografts.

Our experiments demonstrate that alloreactive T cells have a deleterious effect on the morphology of in vitro capillary tubes. These experiments do not focus on the specificity of the T cell–endothelial cell interaction but on the end effect of this interaction. Previous studies have addressed the T-cell aspect of this interaction, showing that endothelial cells can activate T cells as well as be targets of allospecific T cells [7]. We used similar methods in our experiments to derive alloreactive T cells from mice immunized with alloantigens present in the endothelial cells in the capillary-tube assay. This bulk population of alloreactive T cells caused moderate but significant dysfunction of endothelial cells. The selecting of allospecific $CD8^+$ T cells from this population of alloreactive T cells could magnify the dysfunction caused by T cells in the capillary-tube assay.

We also observed that activated alloreactive T cells caused a more severe dysfunction of the endothelial cells in the capillary assay than non-activated alloreactive T cells. These results extend those by other investigators who have shown that T cells activated in the presence of Con A detach endothelial cells in monolayer culture [21]. Both our results with Con A activated and alloreactive T cells and those with IL-2 primed T cells suggest that antigen-specific interaction between T cells and endothelial cells is important but is only a partial requirement for endothelial dysfunction, at least in vitro. Experiments in which T cells without antigen specificity have been shown to cause modulation of integrins on endothelial cells support this notion further [22]. This antigen non-specific T cell–endothelial cell interaction can lead to disengagement of integrins from their ligands in the extracellular matrix, causing detachment of the endothelial cells. Although we have not studied the role of integrins in T cell–endothelial cell interaction, the endothelial dysfunction in the capillary-tube assay in our experiments is, at least partially, from modulation of integrins on the endothelial cells.

The capillary-tube assay is well suited to the study of molecular mechanisms of endothelial dysfunction and detachment that occurs in allograft endothelial dysfunction. Beta-1 integrin modulation, for example, could be an important mechanism of endothelial detachment in endothelial injury and is readily reproduced in the capillary-tube assay. Our preliminary experiments have shown that specific modulation of beta-1 integrins by a blocking antibody, P5D2, leads to complete inhibition of tube formation and disruption of in vitro capillaries. Furthermore, intracellular tyrosine phosphorylation, probably a downstream event of integrin disengagement, seems to play an important role in capillary-tube disruption induced by integrin–ligand disengagement. We have found that endothelial-tube disruption induced by the beta-1 integrin antibody is completely prevented by inhibiting intracellular tyrosine phosphorylation (unpublished data). Thus, the capillary-tube assay described in this report is not only a model for the study of the molecular mechanisms of endothelial dysfunction but is also a potential tool for the devising of strategies to prevent endothelial dysfunction in allografts.

In summary, we have shown that the capillary-tube assay has the potential to be an in vitro model for endothelial dysfunction in allografts. Our experiments suggest that both cellular and soluble mediators of alloinjury cause endothelial dysfunction and that IFN-γ is a key mediator of this effect. Future studies will aim to define the cellular and molecular mechanisms that cause endothelial dysfunction in capillaries and to devise strategies to prevent endothelial damage in allografts.

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