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Human NK Cells Lyse Organ-Specific Endothelial Cells: Analysis of Adhesion and Cytotoxic Mechanisms

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Human organ-specific microvascular endothelial cells (ECs) were established and used in the present study to investigate their susceptibility to natural killer cell line (NKL)-induced lysis. Our data indicate that although IL-2-stimulated NKL (NKL2) cells adhered to the human peripheral (HPLNEC.B3), mesenteric lymph node (HMLNEC), brain (HBrMEC), and lung (HLMEC) and skin (HSmkMEC.2) ECs, they significantly killed these cells quite differently. A more pronounced lysis of OSECs was also observed when IL-2-stimulated, purified peripheral blood NK cells were used as effector cells. In line with the correlation observed between adhesion pattern and the susceptibility to NKL2-mediated killing, we demonstrated using different chelators that the necessary adhesion step was governed by an Mg2+-dependent, but Ca2+-independent, mechanism as opposed to the subsequent Ca2+-dependent killing. To identify the cytotoxic pathway used by NKL2 cells, the involvement of the classical and alternate pathways was examined. Blocking of the Ca2+-dependent cytotoxicity pathway by EGTA/MgCl2 significantly inhibited endothelial target cell killing, suggesting a predominant role for the perforin/granzyme pathway. Furthermore, using confocal microscopy, we demonstrated that the interaction between NKL2 effectors and ECs induced cytochrome c release and Bid translocation in target cells, indicating an involvement of the mitochondrial pathway in NKL2-induced EC death. In addition, although all tested cells were sensitive to the cytotoxic action of TNF, no susceptibility to TRAIL or anti-Fas mAb was observed. The present studies emphasize that human NK cell cytotoxicity toward ECs may be a potential target to block vascular injury. The Journal of Immunology, 2005, 174: 5573–5582.
may result from cytotoxicity toward EC mediated by effector lymphocytes (22–25).

More importantly, it is becoming increasingly clear that NK cells may play a role in the control of angiogenesis (26, 27). In this context, it has been shown that NK cells bind to angiogenic microvessels in established cancer metastases and that vascular endothelial growth factor-A165 appears to stimulate NK cell adhesion to the microvasculature within established cancer metastases (28).

The cross-talk between NK cells and the endothelial system was investigated using porcine or HUVEC cells (20, 23). Despite a series of studies showing the selectivity of the endothelium and the high degree of microenvironmental control of the EC, the interaction between human NK cells and organ-specific ECs remains unknown. The primary aim of the present study was to examine whether a human NK cell line (29) is able to adhere and kill organ-specific human ECs (30). To decipher the way in which NK cells are recruited and bind the endothelial layer to enter the tissue underneath, we studied the adhesion mechanism between NKL and ECs. This could provide a better understanding of the destruction of the vessels necessary for the development of invasive pathologies such as cancer (31). In this paper we show that NKL-mediated EC killing is organ specific and occurs in an IL-2 activation-dependent manner.

Materials and Methods

Cells and culture conditions

All organ-specific EC lines (30) (OSECs) were established in the laboratory (C. Kieda, Centre National de la Recherche Scientifique patent 99-16169) in the purpose of studying the endothelial organo-selectivity. Human microvascular ECs were isolated and immortalized according to the method previously described and patented. Their phenotype was shown to be stable in terms of adhesion molecules and typical EC characteristics (30). The OSEC lines used in this work were human peripheral (HPL-NEC.B3), mesenteric lymph node (HNLMEC), brain (HBrMEC), and lung (HLMCEC) and skin (HSKMEC.2) microvascular ECs. OSECs were cultured in OptiMEM 1 with Glutamax-I (Invitrogen Life Technologies) supplemented with 3% FBS (Biochrom), 40 mg/ml gentamicin (Panpharma), and 0.125 mg/ml fungizone (Invitrogen Life Technologies). OSECs displayed a rounded shape when cultured in basic medium. For most experiments and before any labeling, the cells were detached and trypsinized (Invitrogen Life Technologies), 1% penicillin/streptomycin (Biochrom), and 1% sodium pyruvate (Biochrom).

mAbs and reagents

Anti-cytochrome c, a mouse IgG1 mAb, was purchased from BD Pharmingen, and anti-Fas mAb (F413) was purchased from Sigma-Aldrich. The fluorescent lipophilic dye 3,3′-dihexyloxacarbocyanine iodide 3,3′-dihexyloxacarbocyanine iodide (DiOC₆(3)) was purchased from Molecular Probes, and propidium iodide (PI) was obtained from Sigma-Aldrich.

The PKH26 Red Fluorescent Cell Linker Kit was purchased from Sigma-Aldrich. CFSE was obtained from Molecular Probes. EDTA was obtained from Sigma-Aldrich, and EGTA was obtained from Polysciences. Gelatin 2% solution type B from bovine skin was obtained from Sigma-Aldrich.

Cytotoxicity assay

Trypsin/EDTA-detached OSECs or K562 (NK-sensitive cells; 6 × 10⁴) were incubated for 45 min with 100 μl of Na₅¹⁵²⁵⁵Cr (Amersham Biosciences). After labeling, cells were washed three times with cold medium (10% FBS/RPMI 1640; Invitrogen Life Technologies) and placed in 96-well microplate titers (Falcon; BD Biosciences). Various numbers of effector cells were added to a final volume of 0.2 ml. After a 4-h incubation at 37°C in a 5% CO₂ atmosphere, the plate was centrifuged at 2000 × g for 2 min, the supernatants were harvested, and radioactivities were counted by gamma counter. Maximal or spontaneous release from target cells was obtained by mixing target cells in 0.2 ml of medium (10% FBS/RPMI 1640, v/v) with or without 1 N HCl. The spontaneous release was usually <25% of the total release. The percentage of lysis was calculated using the following equation: lysis (%) = (experimental release - spontaneous release)/(maximum release - spontaneous release)) × 100. In the blocking experiments, EDTA (4 mM) or EGTA (4 mM) and MgCl₂ (2 mM) were added just before the incubation of ¹⁵²⁵⁵Cr-labeled target cells with effector cells.

Labeling experiments

Collagen-detached cells were labeled with Abs specific for TNF-α, TRAIL, and FasRs (30 min at 37°C). After washing with c-PBS, detection by the corresponding FTC secondary Abs was performed for additional 30 min at 4°C. The results were detected by a FACScalibur flow cytometer, and data were processed using CellQuest software (BD Biosciences).

Apoptosis detection

Apoptosis was detected by two-color fluorescence staining. Trypsin-detached ECs (3 × 10⁵) or control cell lines were incubated with DiOC₆(3) (50 μM; Sigma-Aldrich) for 30 min at 37°C to assess mitochondrial membrane potential (32). After two washings in c-PBS, PI (Sigma-Aldrich) was added (1 μg/ml). The samples were read in 1 h on a FACSort (BD Biosciences) flow cytometer (FL1 (DiOC₆): λex = 488 nm; λem = 510 nm; FL2 (PI): λex = 488 nm; λem = 617 nm). Data were processed using CellQuest software (BD Biosciences).

Adhesion test

Static conditions. ECs were seeded into 24-well plate (Falcon) at the desired concentration and cultured for 48 h before the adhesion assay. NKL cells were labeled with PKH26GL red dye (33) and were overlaid onto the EC monolayer in a five lymphoid cells to one EC ratio. The cells were allowed to adhere at 37°C for defined time periods under static conditions. Nonadherent cells were removed by at least three gentle washings. The adhesion was detected by fluorescence microscopy (Axiovert 200M; Zeiss) and then the results were analyzed using AxioVision 3.1 software or was quantified by flow cytometry (FACS LSR; BD Biosciences). Before flow cytometric analysis, all adherent cells were detached using 0.5% trypsin/0.02% EDTA (w/v) solution to disrupt aggregates and provide single cell suspension for quantification. Data were processed using CellQuest Pro software (BD Biosciences). When indicated, divalent cations chelators, EDTA (4 mM) and EGTA (4 mM)/MgCl₂ (2 mM), were added to the medium just before incubating NKL cells with ECs.
Flow conditions. The ECs were seeded onto polystyrene tissue culture slides (Nalge Nunc International) at the desired concentration in normal medium (OptiMEM 1 with Glutamax-I without serum). In coculture experiments, one of the EC lines was first labeled with CFSE. Briefly, trypsin-detached ECs (2 × 10^6) were washed with c-PBS and incubated in CFSE (5 μM) solution in c-PBS for 10 min at 37°C. After three washings with 0.5% (w/v) BSA in c-PBS, cells were mixed in the desired proportion to be seeded with nonlabeled ECs from another line and were cocultured in medium for 72 h.

The cell adhesion flow chamber (Immunetics) was used to perform dynamic cell adhesion assays in a laminar flow environment (under shear stress). Previously labeled with PKH26GL-red (33), NKL2 cells were incubated with ECs at a ratio of 5:1 for the indicated time. After washing with c-PBS, cells were trypsin-detached and analyzed by a FACs LSR; the results were plotted as the number of NKL cells per one EC. Results, expressed as the mean and SD, were calculated from triplicate determinations in one representative experiment of three performed.

Confocal microscopic analysis

The ECs were seeded onto gelatin-coated (0.2% in distilled water) microcoverglasses (Erie Scientific) at the desired concentration and were allowed to grow in culture conditions for 48 h. PKH26GL-labeled NKL cells (33) were overlaid onto the ECs at a 10 lymphoid cells to one EC ratio. After a 4-h incubation, the coverglasses were washed once with c-PBS and fixed with a 4% paraformaldehyde (w/v) solution (Sigma-Aldrich) in c-PBS for 60 min at room temperature. Cells were washed three times with c-PBS. SDS (0.1% in c-PBS; Bio-Rad) was used to permeabilize the cells for 10 min at room temperature. After three washings with c-PBS, free aldehyde residues were blocked by incubation with 10% FBS solution (v/v) in c-PBS for 20 min. Then cells were allowed to react with Ab against cytochrome c (BD Pharmingen) or Bid (Santa Cruz Biotechnology) for 60 min at room temperature. Cells were washed three times with c-PBS and incubated with Alexa 488 (λex = 495 nm; λem = 519 nm)-conjugated goat anti-mouse IgG or Alexa 488-conjugated goat anti-rabbit IgG secondary Abs (Molecular Probes). Micro-coverglasses were washed and mounted on slides using a drop of Vectashield antiphoto-bleaching reagent (Vector Laboratories). Cells were analyzed with a confocal microscope imaging system (MRC-1024; Bio-Rad) equipped with an Optiphot epifluorescence microscope (Nikon) and a Planapo objective (numerical aperture, 1.4). A krypton/argon laser was tuned to produce both 488 nm (fluorescein excitation) and 568 nm (rhodamine excitation) wavelengths. The images were recorded under a Kalman filter (an average of seven images) and were enhanced with Photoshop software (Adobe Systems).
Results

Adhesion of NKL cells to organ-specific EC lines

We used the human NK cell line, NKL in these studies to overcome variability between fresh NK cells obtained from healthy donors. We first investigated the capacity of IL-2-starved (NKL1) or IL-2-stimulated (NKL2) NKL to adhere to organ-specific ECs (OSECs) from distinct origins. Fig. 1A indicates that under static conditions, IL-2 dramatically enhanced NKL cell adhesion to OSECs. As shown in this figure, in the absence of IL-2 stimulation (NKL1), the adhesion of NKL remained very low over the whole incubation period (from 0.5 to 30 min), suggesting that NKL1/OSECs recognition did not trigger the adhesion molecule cascade. This phenomenon appeared to be organ specific, because lymphoid organ-derived ECs (from peripheral HPLNEC.B3 and mesenteric HMLNEC lymph nodes) were more efficient NKL2 binders (3-fold) than peripheral tissue-derived ECs. As shown in this figure, in the absence of IL-2 stimulation (NKL1), the adhesion of NKL remained very low over the whole incubation period (from 0.5 to 30 min), suggesting that NKL1/OSECs recognition did not trigger the adhesion molecule cascade.

This phenomenon appeared to be organ specific, because lymphoid organ-derived ECs (from peripheral HPLNEC.B3 and mesenteric HMLNEC lymph nodes) were more efficient NKL2 binders (3-fold) than peripheral tissue-derived ECs. NKL1 adhered very poorly, but preferentially, to lung-derived ECs (HLMEC), as illustrated in Fig. 1B. The selectivity of NKL2 cells compared with NKL1 (Fig. 1A) demonstrates the role of IL-2 and the activation dependency of NKL cells to give them the ability to recognize and bind efficiently to the endothelium. The above observations concerning IL-2 activation and the organ specificity of NKL/endothelium recognition were confirmed using fluorescence microscopy, as shown in Fig. 1B by the adherence of NKL2 cells (orange) to an EC monolayer, as shown in the control line (Fig. 1B).

Specificity of EC recognition by NKL2 demonstrated in flow conditions

Because adhesion experiments performed under shear stress are more selective, we cocultured ECs from two different lines before submitting them to NKL2 cell adhesion under flow. EC were distinctly fluorescently labeled before the coculture to distinguish them, as shown in Fig. 2A (HSkMEC.2, CFSE-labeled/HMLNEC-nonlabeled) and 2C (HMLNEC, CFSE-labeled/HSkMEC.2-nonlabeled). NKL2 cells were then allowed to roll and to adhere by dynamic flow running on the EC layer to assess the selectivity of NK/EC-type binding. Fig. 2 shows the NKL2 adhesion performed under controlled flow conditions on such a coculture of the two EC lines tested. NKL2 were able to discriminate the mesentery-derived cells from the skin-derived ones (Fig. 2, B and D), confirming the static adhesion assay data. Indeed, as shown in Fig. 2B, three NKL2 cells bound to one HMLNEC (nonlabeled) cell, and only one NKL2 bound to one HSkMEC.2 (labeled) cell. The possible influence of the fluorescent marker of EC on adhesion could be excluded, as shown in the control experiment (Fig. 2, C and D) in which the second EC type was labeled. Fig. 2D shows three NKL2 cells bound to one HMLNEC (labeled) and none to HSkMEC.2 (nonlabeled) cells. Such conditions did not only confirm the selectivity observed in static adhesion assays (Fig. 1A), but further enlighten the discriminative character of the organ-specific ECs toward circulating leukocytes.

Additional tests performed in flow conditions using HPLNEC.B3 and HSKMEC.2 cells demonstrated the same type of clear-cut selectivity for HPLNEC.B3 (data not shown) as opposed to HSKMEC.2, confirming the static adhesion results.

Human IL-2-stimulated NKL cells kill OSEC targets

Based on the observed adhesion between NKL2 cells and OSECs, the sensitivity of the latter to IL-2-activated NKL-induced cytotoxicity was examined using the standard chromium release assay. As shown in Fig. 3A, all ECs were significantly and efficiently killed by NKL2. Although resting purified peripheral blood NK
did not show any killing activity (data not shown), when stimulated with IL-2 a more pronounced lysis, compared with that induced by NKL2, was observed (Fig. 3B).

**Susceptibility of OSECs to death receptor ligands**

NK cells use the perforin and granzyme pathway to kill their targets, but also use the alternate pathway, involving death receptors and their ligands. Therefore, we examined the susceptibility of these cells to killing by death receptor ligands. Fig. 4 illustrates the typical death observed after TNF, TRAIL, and anti-Fas treatments. Using DIOC/PI staining and flow cytometric assessment, a selective susceptibility toward TNF-α cytotoxic action was detected compared with Apo2-TRAIL and anti-Fas effects. Although all cells were killed after a 72-h TNF-α treatment, they were resistant to anti-Fas- or TRAIL-dependent apoptosis (after 24 and 48 h). These results corroborate the expression of death receptors by OSECs. Indeed, flow cytometric analysis revealed the expression of TNFR type 1 by all cell lines tested, whereas Apo2-TRAIL and FasR were not detected (data not shown).

**Triggering of cytochrome c release in OSECs targets upon conjugation with NKL2 cells**

Although granzyme B can directly activate caspases, it induces apoptosis predominantly via Bid cleavage, mitochondrial outer membrane permeabilization, and cytochrome c release. In addition, evidence has been provided indicating that activation of the apoptotic machinery through activation of caspase 8 and mitochondria was directly connected by truncated Bid protein, resulting in the release of proapoptotic factors from mitochondria. To determine the possible involvement of mitochondria in OSEC-induced NK killing, we assessed the subcellular localization of Bid and cytochrome c by confocal microscopy. For this purpose, OSECs were incubated with NKL2 (4-h incubation at the ratio of 10 NKL2 to one EC) and subjected to immunostaining for Bid and cytochrome c. A punctuated cytoplasmic staining pattern showed mitochondrial localization of cytochrome c, whereas a diffuse staining pattern indicated the mitochondrial release of cytochrome c. The data presented in Fig. 5A show that cytochrome c was released from the mitochondria intermembrane space to the cytoplasm in ECs upon coculture with NKL2 cells. Fig. 5B shows that in the two OSECs tested (HBrMEC and HMLNEC), Bid was cleaved and translocated from the cytosol to the mitochondria when those cells were incubated with NKL2. These data clearly indicate that NKL2 cells are able to activate the mitochondrial pathway to kill OSEC targets.

**Killing of OSECs is predominantly mediated by granzyme B**

To examine the mechanisms used by NKL2 to mediate their cytotoxic activity toward OSECs, blocking experiments were performed in the presence of EGTA/MgCl₂ (used to inhibit the Ca²⁺/H₁₁₀₀₁-dependent perforin/granzyme-mediated lysis). The results presented in Fig. 6 clearly show that the cytotoxicity induced by

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**FIGURE 4.** Effects of TNF-α, TRAIL, and anti-Fas on EC viability. Apoptosis was assessed using DIOC₆/PI staining by flow cytometry. ECs were incubated with TNF-α (250 ng/ml; 72 h), Apo2TRAIL (1 μg/ml; 24 h), and anti-FAS (24 h). After 30-min incubation with DIOC₆ (50 μM) at 37°C, the samples were washed with c-PBS. PI (1 μg/ml) was added just before acquisition by FACSsort. Apoptosis efficiency is indicated as a percentage, as are control values.
FIGURE 5. NKL2 binding triggers the mitochondrial pathway in OSEC target cells. A, Cytochrome c release by ECs after interaction with NKL2. The EC lines (HBrMEC and HMLNEC) were seeded onto gelatin-coated glass and cultured for 72 h. PKH26GL-labeled NKL2 cells (ratio 10:1) were overlaid and incubated for 4 h at 37°C. After washing and fixation, cells were stained with Ab against cytochrome c and secondary Alexa 488-conjugated Abs. The images were collected using a confocal microscope imaging system (MRC-1024; Bio-Rad). Similar results were observed with all tested ECs. B, Bid translocation in ECs upon interaction with NKL2. For incubation conditions, see above. Staining was achieved with anti-Bid Ab and secondary Alexa 488-conjugated Abs. The images were collected using a Zeiss Axiovert 200 inverted fluorescence microscope. Similar results were observed with all tested ECs.
NKL2 was largely inhibited after Ca\(^{2+}\) chelation, indicating that these killer cells use the granule exocytosis pathway to kill OSEC targets.

**Distinct divalent cation requirement for NKL2 adhesion and subsequent triggering of OSEC lysis**

A comparative analysis of the effect produced by distinct chelating agents, EDTA vs EGTA in the presence of MgCl\(_2\), toward NKL2 cell adhesion to OSECs was performed to assess the molecular mechanisms that control adhesion and lysis. Using EGTA/MgCl\(_2\), we demonstrated that the calcium-dependent pathway was associated with promotion of the lytic effect. Because this cannot be achieved without the prior adhesion/recognition step, we attempted to distinguish the two steps on the basis of their divalent cation susceptibility. Adhesion tests were performed, during which the effect of EDTA (Ca\(^{2+}\) and Mg\(^{2+}\) chelating agent) compared with that of EGTA/MgCl\(_2\) (preferentially chelating Ca\(^{2+}\)) was examined. EDTA strongly inhibited the NKL2 cell adhesion to OSECs, as revealed by flow cytometry (Fig. 7A) and as evidenced by inverted and fluorescence microscopy (Fig. 7B). This was true for both NKL2 efficient adhesion as well as for low NKL1 adhesion (data not shown), which are both sensitive to EDTA.

EDTA, which is known as a chelator of different divalent cations (18, 34), is able to bind both calcium and magnesium ions with almost the same affinity at neutral pH: log K = 7.27 and log K = 5.37, respectively; whereas EGTA in the presence of Mg\(^{2+}\) is suitable for demonstrating calcium cation involvement alone (due to its higher association constant for calcium: log K = 6.68 compared with magnesium log K = 1.61 at pH 7) (35).

Fig. 7B shows that in contrast to cell killing, incubation with EGTA/MgCl\(_2\) did not inhibit their adhesion. Consequently, Ca\(^{2+}\) chelation did not affect the adhesion in contrast to Mg\(^{2+}\) chelation. This indicates that selectins and other Ca\(^{2+}\)-dependent lectins were not involved in NKL2/OSEC recognition, whereas integrins, which are the main adhesion molecules, of which activity is Mg\(^{2+}\) dependent, might participate to NKL2 adhesion to organo-selective ECs.

**Discussion**

ECs are primary targets of immunological attack, and study of the interactions between the endothelium and immune cells has significantly expanded during the past decade. Among others, xenograft rejection studies showed that several human leukocyte subsets, including NK, CTL, and polymorphonuclear neutrophils, participate in the cellular immune response directed against vascularized pig-to-human xenografts (36). NK cells are one of the most important mediators in xenogeneic immune responses (37). They were reported to adhere to xenogeneic porcine ECs, and evidence from both in vitro as well in vivo models suggests their role in the recognition and damage of porcine xenogeneic tissues (38).

Most of the in vitro models that provided information about NK cells and EC interactions involved HUVEC (23, 24, 39). These studies are clearly limited by the lack of organ-specific and microenvironmental factors (40–42). The objective of this study was to elucidate the interactions that govern human NK organ-specific EC adhesion and to dissect the NK-mediated killing process of ECs. Experiments were first aimed at investigating the adhesion process in our model.

The present studies indicate that only IL-2-activated NKL were able to adhere to ECs, thus IL-2 may promote NK cells to adhere to ECs. This is consistent with models in which endothelium killing by NK cells is activation state dependant (43) and further corroborates previous reports demonstrating that IL-2 stimulation is necessary for proper activity of NK cells (44–46). IL-2-enhanced adherence to selective targets is confirmed in this work using flow adhesion methods. Although previous work reproducing the blood flow shear stresses showed that IL-12 stimulation of NK cells promotes their adhesion to endothelial β selectin (47), Schneider et al. (38) described the role played by integrins in that dynamic process. Using IL-2 stimulated NKL cells (NKL2) and immortalized EC lines as a model for organo-specific barriers (21, 30, 41, 42) by both stable and flow adhesion assays, we could show in this study that the recognition and adhesion are highly selective and correlate with the killing process. In this regard, flow conditions appear to discriminate more than static adhesion assay. Furthermore, we showed that the recognition was independent of Ca\(^{2+}\); consequently, it did not involve selectins, whereas the Mg\(^{2+}\) requirement indicated the integrin involvement.

To determine whether such interactions may result in the killing of organ-specific endothelial targets, cytotoxic assays were performed. We provide experimental data indicating that human NKL cells efficiently kill organ-specific ECs. This killing occurs only when NKL and purified NK cells are stimulated with IL-2, suggesting that adhesion and killing require NK stimulation to increase their adhesive features and their lytic potential. Using purified peripheral blood NK cells, we demonstrated that these effectors were also able to kill OSECs only after IL-2 activation. Current evidence supports the idea that ECs present at the vascular walls as well as specialized endothelial venules play not only a critical role in the homing and recruitment of immune cells, but also influence the outcome of the immune response. In this regard, porcine aortic ECs were shown to activate human dendritic cells and subsequently transactivate naïve T cells through costimulation and cytokine generation (48). In addition, EC play a critical immune regulatory role in the course of Ag presentation by MHC class II to transmigrating T cells (49). In the same context, Waldman et al. (50) have shown that allograft endothelium harboring CMV has the potential to activate host T cells, resulting in cytokine production and increased alloimmunogenicity. These observations support the view that ECs play a role in both the homing and recruitment of immune cells and the regulation of immune response through the factors they release and their physical interaction with immunocompetent cells. IL-2-stimulated NK cells have been used in immunotherapy of cancer (51). In most cases the activation of NK resulted in the induction of vascular leak syndrome (52). Although, the mechanism of IL-2-induced vascular leak syndrome is still poorly understood, it has been suggested that TNF-α may play a central role in mediating this syndrome associated with IL-2 toxicity (53). IL-2 may have both direct (54) and indirect effects (55). This is consistent with the pathogenesis of EC complexity damage involving activation or damage to ECs and leukocytes, release of cytokines and inflammatory mediators, and
alterations in cell-cell and cell-matrix adhesion and in cytoskeleton function.

Cells of both innate and adaptive immune systems have been implicated, but no definitive conclusions have been reached concerning their respective roles. Recently, NK cells have been shown to be critical for IL-2-induced pulmonary vascular leak syndrome in a murine model (56).

We have shown in the course of these studies that organ-specific ECs were not sensitive to Fas and TRAIL pathways, but exhibit a significant sensitivity to the cytotoxic action of TNF, confirming the known effect of this cytokine on vascular destruction (57) and its possible antiangiogenic effect (58). Whether NK kill organ-specific ECs through TNF release has yet to be determined. However, our studies implicate the major cytotoxic pathway involving granzyme B as a potential component associated with execution of the NK lytic program. This is in agreement with the report by Matter et al. (59) indicating that the xenogeneic human NK cytotoxicity against porcine ECs is perforin/granzyme B dependent. It has been shown that fractalkine plays an important role not only in the binding of NK cells to ECs, but also in NK cell-mediated endothelium damage (24). How ECs activate NK cells and, particularly, the role of chemokines in the cross-talk between NK and ECs remain issues that will be addressed in a future investigation.

Given the fact that through their interactions, ECs and NK cells modulate each other through cytokine release, it is tempting to speculate that the NK/EC interaction may play a role in modulating EC function and influence the outcome of both adaptive and innate immunity as well. It should be underlined, therefore, that NK may have

**FIGURE 7.** Divalent cation requirement for NKL2 adhesion. A. Inhibition of NKL2 cell adhesion toward EC lines by EDTA. PKH26GL-labeled NKL2 cells were incubated with ECs at a 5:1 ratio for 30 min in the presence or the absence of EDTA (4 mM) or EGTA (4 mM/MgCl₂ (2 mM), detached, and analyzed on a FACS LSR. The results reflect the number of NKL2 cells per one EC (mean and SD) and were calculated from triplicate determinations in one representative experiment of five performed. B. Distinct effects of Ca²⁺ vs Mg²⁺ in the adhesion process. PKH26GL-labeled NKL2 cells were incubated with ECs for 30 min at a ratio of 5:1 in the presence or the absence of EDTA (4 mM) or EGTA (4 mM)/MgCl₂ (2 mM). Data were analyzed with a Zeiss Axiovert 200 inverted fluorescence microscope.
complex effects, including the damage of normal vessel (leak syndrome), the damage of pathological vessels, and a cytotoxic activity toward transformed cells. It is important to take these parameters into account as well as their influence on the angiogenesis process because of the influence of the latter on activated NK cell recruitment (60). This was shown to involve comparable adhesion processes as shown by the mechanisms described in the present work, which consequently may help in the design of new cell therapy protocols.

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Disclosures

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References


