T-Lymphocyte–Derived Tumor Necrosis Factor Exacerbates Anoxia-Reoxygenation–Induced Neutrophil–Endothelial Cell Adhesion

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Abstract—The overall objective of this study was to determine whether T lymphocytes can modulate the increased neutrophil adherence and upregulation of endothelial cell adhesion molecules in human umbilical vein endothelial cells (HUVECs) exposed to anoxia/reoxygenation (A/R). HUVEC monolayers were exposed to 60 minutes of anoxia, followed by 4 hours of reoxygenation in the absence or presence of human T lymphocytes. The A/R-induced neutrophil adhesion was significantly enhanced when T lymphocytes and HUVECs were cocultured for the first 45 minutes of reoxygenation. This was accompanied by a more pronounced increase in E-selectin expression. When T lymphocytes were cocultured with HUVECs by use of inserts that prevented direct cell-cell contact, a comparable A/R-induced enhancement of neutrophil adhesion and of E-selectin expression was observed, indicating that soluble factors produced by T lymphocytes mediate the exaggerated A/R-induced inflammatory responses. Treatment with either an anti– tumor necrosis factor-α antibody or catalase attenuated the T-cell–mediated responses in postanoxic HUVECs. Moreover, the T-cell–mediated neutrophil adhesion response was mimicked by exposure of naive HUVECs to H₂O₂. These findings indicate that H₂O₂ produced by postanoxic endothelial cells stimulates T cells to produce tumor necrosis factor-α, which in turn elicits endothelial cell adhesion molecule expression and a corresponding increase in neutrophil adhesion. (Circ Res. 2000;86:205-213.)

Key Words: tumor necrosis factor-α ■ hydrogen peroxide ■ E-selectin ■ T lymphocyte ■ neutrophil

The phenomenon of ischemia/reperfusion (I/R) has been implicated in the microvascular and parenchymal cell injury associated with several pathological conditions, including gastric ulcer formation, multiple organ failure, cancer metastasis, and organ transplantation. It is well known that the microvasculature is highly sensitive to I/R and that the hyperadhesiveness of leukocytes to endothelial cells contributes to I/R-induced microvascular dysfunction and tissue injury. We and others have used a simple in vitro model wherein endothelial cell monolayers are exposed to anoxia/reoxygenation (A/R) in order to mimic the microvascular dysfunction that is elicited by I/R in vivo. Studies using this model have revealed that the responses of cultured endothelial cells to A/R closely parallel the responses observed in postischemic microvessels, including enhanced oxidant production, activation of nuclear transcription factors, expression of endothelial cell adhesion molecules (CAMs), increased adhesivity to neutrophils, and loss of endothelial barrier function. These in vitro models have also provided a detailed characterization of the contribution of different leukocyte and endothelial cell adhesion glycoproteins to the elevated neutrophil–endothelial cell adhesion elicited by A/R.

Although A/R-exposed endothelial cells have provided considerable insight into the mechanisms that account for the inflammatory responses observed in microvessels exposed to I/R, there are limitations inherent in this approach that may limit its ability to accurately simulate the more complex in vivo responses to I/R. An important limitation of the in vitro models relates to their simplicity and the absence of auxiliary cell types (eg, mast cells, macrophages, platelets, and lymphocytes) that may modulate the responses of neutrophils and/or endothelial cells to the stimulatory effects of A/R. There is evidence that implicates lymphocytes in the pathogenesis of I/R injury in tissues such as the liver. Reperfusion of the ischemic liver is associated with the sequestration of circulating lymphocytes in the hepatic microcirculation, where they appear to aggravate the injury response to I/R. The protective action of immunosuppressive agents like cyclosporine and FK506 or antibodies that deplete CD4+ T lymphocytes in animal models of I/R-induced liver injury and inflam-
information provides further support for the participation of T cells in I/R injury.

Although these observations implicate T cells in I/R injury, it remains unclear whether lymphocytes directly mediate tissue injury or indirectly influence the I/R response by modulating the activation state of endothelial cells and/or neutrophils. Recently, we demonstrated that postanoxic endothelial cells sustain the adhesion of T lymphocytes at 8 hours after reoxygenation, indicating that T lymphocytes per se are able to bind to hyperadhesive postanoxic endothelial cells, where they may contribute to the reoxygenation-induced injury response. There is circumstantial evidence in the literature suggesting that T cells may also exacerbate the neutrophil-endothelial interactions that are normally elicited by I/R. For example, it has been shown that neutrophil accumulation in the postischemic liver is greatly diminished in mice depleted of CD4 T cells. This observation, coupled with in vitro and in vivo studies demonstrating a dependence of endothelial CAM expression on T lymphocytes, suggests that T cells could determine the magnitude of CAM expression (and the consequent recruitment of adherent neutrophils) on endothelial cells exposed to I/R (or A/R). Hence, the overall objective of the present study was to determine whether T lymphocytes can influence the intensity of the neutrophil–endothelial cell adhesion and of the endothelial CAM expression that is observed in HUVECs exposed to A/R. Because T cells proved to exert a significant influence on the endothelial cell responses to A/R, we also investigated the mechanisms that underlie this modulating influence of T lymphocytes on postanoxic endothelial cells.

Materials and Methods

Materials

Histopaque 1077, catalase (CAT), and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. Tissue culture supplies were purchased from Clonetics, Biomedical Technologies, or HyClone Laboratories. WEB 2086 was obtained from Boehringer. Monoclonal antibodies (mAbs) directed against intercellular adhesion molecule-1 (ICAM-1), P-selectin, and E-selectin were gifts from Dr Donald Anderson, Pharmacia-Upjohn Laboratories.
Cell Culture

Human umbilical vein endothelial cells (HUVECs) were harvested from freshly discarded human umbilical cords by collagenase perfusion as previously described. The cells were grown in endothelial growth medium supplemented with bovine brain extract in 5% CO2. Endothelial cells were characterized by positive labeling with acetylated LDL labeled with fluorescently labeled acetylated LDL (Dil-Ac-LDL) or mouse anti-human factor VIII. Passage 1 cultures were used for the studies.

Isolation of Neutrophils and Lymphocytes

Human neutrophilic polymorphonuclear cells were isolated from venous blood of healthy adults by using standard dextran sedimentation and gradient separation on Histopaque 1077. Total lymphocytes (T and B lymphocytes) and the T-lymphocyte population were isolated by the method of Berney and Atkinson.

A/R Protocol

The in vitro model of A/R used in the present study is similar to that previously reported and has been used extensively in our laboratory. Confluent HUVEC monolayers were exposed to anoxia by incubation in a Plexiglas chamber that was continuously purged (1 L/min) with an anoxic gas mixture (93% N2/5% CO2/2% H2). Reoxygenation was performed by exposing endothelial monolayers to room air in the CO2 incubator. Control cells were exposed to normoxia (21% O2/5% CO2/74% N2). In some experiments, naive HUVEC monolayers were exposed to H2O2 (0.05 or 0.1 mmol/L) with or without T lymphocytes.

Adhesion Assays

Neutrophil adhesion to endothelial cells (ratio of 10:1) was performed with the use of 51Cr-labeled neutrophils as previously described. Each adhesion assay was performed 4 hours after reoxygenation. The effect of T lymphocytes on neutrophil adhesion was assessed by preincubation of T cells with HUVECs (ratio of 5:1) for 45 minutes after reoxygenation before neutrophil adhesion assay. In some experiments, T lymphocytes were separated from HUVEC monolayers by 0.45-μm Millipore filters. In other studies, supernatants from postanoxic endothelial cells were collected and added to naive HUVEC monolayers coincubated with T lymphocytes. The role for superoxide, H2O2, or platelet-activating factor (PAF) was tested by pretreatment of HUVEC monolayers with blocking levels of the respective antioxidant enzymes, SOD (1000 U/mL) or CAT (1000 U/mL), or a PAF receptor antagonist (WEB 2086, 10 μmol/L) at concentrations that we and others have previously shown to...
attenuate reactive oxygen species–mediated or PAF-mediated neutrophil adhesion to endothelial cells. The role for TNF-α and E-selectin on neutrophil adhesion was examined in the presence of blocking doses of mAbs for TNF-α (10 μg/mL) and E-selectin (20 μg/mL). Endothelial CAM expression was performed by ELISA with the use of the respective primary antibodies (4 μg/mL) for either ICAM-1, P-selectin, or E-selectin. TNF-α levels in media from A/R-conditioned HUVEC monolayers without or with coculture with T lymphocytes were quantified by use of a human TNF-α ELISA kit.

Statistical Analysis

All values are expressed as mean±SE. Data were analyzed by 1-way ANOVA with Bonferroni corrections for multiple comparisons or the Fisher protected least significant difference test. An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Figure 1 illustrates the effect of total lymphocytes (T and B lymphocytes) on neutrophil adhesion to HUVEC monolayers exposed to normoxia or A/R. The results show that in the absence of T cells, exposure of endothelial cells to 60 minutes of anoxia and 4 hours of reoxygenation enhances neutrophil adhesion to HUVEC monolayers compared with normoxia-exposed cells, consistent with previous observations.3 Coculture of endothelial cells with peripheral blood total lymphocytes resulted in a greater increase in neutrophil adhesion under A/R, but not normoxic, conditions. To determine the lymphocyte population that contributed to the exaggerated response during A/R, we fractionated T lymphocytes from B cells and performed the adhesion assay at 4 hours after reoxygenation. Figure 2 shows that the adherence of neutrophils to normoxic HUVEC monolayers is unaffected by T lymphocytes, but these cells exacerbated the A/R-induced neutrophil adhesion to HUVECs to the same extent as that elicited by mixed T- and B-cell populations (see Figure 1), thus confirming that T lymphocytes are the primary contributors to the exaggerated neutrophil adhesion response during A/R.

To evaluate whether the enhanced neutrophil adhesion was dependent on contact between T lymphocytes and HUVECs, T lymphocytes were separated by 0.45-μm Millipore filters in culture inserts from HUVEC monolayers grown in 24-well plates. The results in Figure 3 shows that separation of T lymphocytes from HUVEC monolayers elicited an A/R-induced neutrophil adhesion response that was quantitatively similar to that observed in the absence of inserts (see Figure 2), suggesting that T-lymphocyte–derived soluble factors are responsible for the exacerbated neutrophil–endothelial cell adhesion after A/R.

There are several reports that demonstrate the ability of T lymphocytes to produce and release TNF-α, a cytokine that can engage with specific receptors on endothelial cells to induce the transcription-dependent production of CAMs, such as E- and P-selectin and ICAM-1.18–20 To determine whether postanoxic endothelial cells elicit the release of TNF-α by T lymphocytes, we quantified the levels of TNF-α in the incubation medium of A/R-conditioned HUVEC monolayers in the absence or presence of T lymphocytes and with or without treatment with the superoxide dismutase (SOD) and catalase (CAT).

Figure 7. Effect of SOD, CAT, and PAF receptor antagonist (WEB 2086) on T-lymphocyte–enhanced neutrophil adhesion. HUVEC monolayers and T lymphocytes were cocultured in the cell culture insert system under normoxic or A/R conditions as described in Figure 3. SOD, CAT, and WEB 2086 were added to HUVEC monolayers before exposure to A/R. After 45 minutes of coculture, T lymphocytes were removed, and neutrophil adhesion assay was performed. Each value represents mean±SE of 4 experiments performed in triplicate. *P<0.01 compared with A/R; #P<0.01 compared with A/R+T lymphocyte.

Figure 8. Effect of T lymphocytes on H2O2-induced neutrophil adhesion to HUVEC monolayers. HUVEC monolayers were exposed to H2O2 (0.05 or 0.1 mmol/L) in the absence and presence of T lymphocytes that were separated from the HUVEC monolayers by a 0.45-μm filter. After 45 minutes, T lymphocytes were removed, and 51Cr-labeled neutrophils were added to HUVEC monolayers after 4 hours of H2O2 exposure. Each value represents mean±SE of 3 experiments performed in triplicate. *P<0.05 and **P<0.0001 compared with control; #P<0.01 compared with other H2O2 (0.1 mmol/L) plus T-cell coinubation values.
without CAT treatment. The results (Figure 4) show that TNF-α concentrations were significantly increased in A/R-conditioned media when cocultured with T lymphocytes, consistent with T-cell release of the cytokine. Treatment with CAT completely blocked this increase in TNF-α in the media, suggesting that T-cell release of TNF-α was mediated by H₂O₂. To addressed the possibility that T-lymphocyte–derived TNF-α mediates the stimulatory effect of these cells on A/R-induced neutrophil–endothelial cell adhesion, T lymphocytes and HUVECs were incubated in the cell insert coculture system in the presence of a monoclonal antibody directed against TNF-α. Figure 5 demonstrates that anti–TNF-α completely abrogated the T-cell–mediated increase in A/R-induced neutrophil adhesion. Interestingly, anti–TNF-α exerted minimal effects on the neutrophil adhesion response elicited by A/R alone (Figure 5). These results suggest that the enhancement of A/R-induced neutrophil adhesion response mediated by T-lymphocyte–derived TNF-α, whereas the cytokine does not contribute to the neutrophil adhesion induced by A/R in the absence of T cells.

Our previous studies have shown that A/R enhances endothelial cell production and liberation of H₂O₂ and PAF during the early stages of reoxygenation (within 30 minutes), which contributed to the neutrophil–endothelial cell adhesion observed during both phase 1 (30 minutes) and phase 2 (4 hours) after reoxygenation. Moreover, Yoshida et al² have reported that both CAT and a PAF antagonist ameliorate the early-phase (within 30 minutes) neutrophil adhesion to naive HUVEC monolayers exposed to media obtained from A/R-conditioned endothelial cells. On the basis of these observations, we assessed the influence of A/R-conditioned media on T-lymphocyte stimulation of A/R-induced neutrophil adhesion in phase 2 (4-hour reoxygenation). This was accomplished by treating HUVEC–T-lymphocyte cocultures in inserts with media obtained from endothelial cells exposed to 60 minutes of anoxia followed by 45 minutes of reoxygenation. The results of these experiments are summarized in Figure 6. In the absence of T cells, media from both normoxic or A/R-conditioned HUVECs did not increase neutrophil adhesion to naive HUVEC monolayers. However, media from A/R-conditioned HUVECs significantly increased neutrophil adhesion to naive HUVEC monolayers cocultured with T lymphocytes. This observation suggests that soluble factors derived from A/R-exposed HUVEC monolayers contribute to the T-lymphocyte–mediated enhancement of neutrophil–endothelial cell adhesion after A/R. Because A/R was shown to enhance the production and liberation of H₂O₂ and PAF during the early stages of reoxygenation,³ we investigated the role of H₂O₂ or PAF in the enhanced neutrophil adhesion by T lymphocytes. As shown in Figure 7, the enhanced adhesion induced by coculture of T lymphocytes with HUVEC monolayers was attenuated by CAT but not by SOD or by a PAF receptor antagonist. Moreover, the A/R-induced T-cell–mediated neutrophil adhesion response was mimicked by treatment of naive HUVEC monolayers with H₂O₂ (0.05 mmol/L and 0.1 mmol/L) and was abrogated by CAT (Figure 8). Taken together, these results are consistent with a role for endothelial cell–derived H₂O₂ in T-cell activation.

Figure 9. Surface expression of P-selectin (A), E-selectin (B), and ICAM-1 (C) on A/R-exposed HUVEC monolayers cocultured with T lymphocytes. HUVEC monolayers were exposed to 60 minutes of anoxia (or normoxia) and then reoxygenated in the absence and presence of T lymphocytes. After 45 minutes of coculture, T lymphocytes were removed, and endothelial CAM expression was determined at 4 hours after reoxygenation. Each value represents mean ± SE of 3 experiments performed in triplicate. *P<0.05, **P<0.01, and ***P<0.001 compared with normoxia (control); #P<0.01 compared with A/R.
To define the molecular determinants of the T-lymphocyte–mediated enhancement of neutrophil–endothelial cell adhesion on postanoxic HUVECs, we quantified the surface expression of endothelial CAMs that are known to mediate neutrophil–endothelial cell adhesion. Figure 9 summarizes the changes in surface expression of different endothelial CAMs on A/R-exposed HUVEC monolayers cocultured with or without T lymphocytes. The data show that 60 minutes of anoxia followed by 4 hours of reoxygenation resulted in increased expression of P-selectin, which was unaffected by coculturing with T lymphocytes (Figure 9A). E-selectin was not expressed on unstimulated endothelial cells and was minimally affected by A/R in the absence of T lymphocytes. The data show that 60 minutes of anoxia followed by 4 hours of reoxygenation resulted in increased expression of P-selectin, which was unaffected by coculturing with T lymphocytes (Figure 9A). E-selectin was not expressed on unstimulated endothelial cells and was minimally affected by A/R in the absence of T lymphocytes. However, E-selectin expression was significantly elevated during A/R exposure (Figure 9B). Unlike E- and P-selectin, the constitutive surface expression of ICAM-1 was high in HUVECs and was further increased by A/R exposure (Figure 9C), consistent with our previous studies. To test whether T-lymphocyte–derived soluble factors, such as TNF-α, mediate the enhanced expression of endothelial CAMs, we cocultured T cells with HUVECs in the cell culture inserts that prevented direct contact of the 2 cell populations. The data (Figure 10) show that the expression of all 3 endothelial CAMs was similar to that observed in HUVEC monolayers, wherein the T lymphocytes were in direct contact with the endothelial cells. The surface expression of all 3 adhesion molecules was significantly increased by A/R, but only E-selectin expression was further enhanced in the presence of T lymphocytes. Collectively, these results suggest that the T-cell–mediated enhancement of A/R-induced neutrophil–endothelial cell adhesion results from an increased expression of E-selectin and that T-lymphocyte–derived soluble factors elicit these responses.

To address the possibility that E-selectin expression is mediated by T-lymphocyte–derived TNF-α and endothelial cell–derived H₂O₂, we quantified the surface expression of E-selectin on postanoxic HUVECs in the presence of anti–TNF-α or CAT. The results in Figure 11 show that the increased A/R-induced E-selectin expression caused by T lymphocytes was completely abrogated by the monoclonal antibody directed against TNF-α. CAT treatment similarly blocked the T-lymphocyte–mediated E-selectin expression, whereas the combined treatment with anti–TNF-α and CAT was without additional effects. To verify that the T-cell exacerbation of neutrophil adhesion to HUVECs was, in fact, mediated by the upregulation of E-selectin, experiments were performed in the presence of a blocking mAb directed against E-selectin. The results are illustrated in Figure 12. In the absence of T lymphocytes, A/R-induced neutrophil adhesion response was significantly attenuated by anti–E-selectin, consistent with a role for E-selectin in the postanoxic neutrophil–endothelial cell interactions as we previously de-

Figure 10. Effect of T-lymphocyte–derived soluble factors on surface expression of P-selectin (A), E-selectin (B), and ICAM-1 (C) on A/R-exposed HUVEC monolayers cocultured with T lymphocytes. HUVEC monolayers cultured in cell culture inserts were exposed to 60 minutes of anoxia (or normoxia) and then reoxygenated in the absence and presence of T lymphocytes that were separated from the HUVEC monolayers by a 0.45-μm filter. After 45 minutes, T lymphocytes were removed, and endothelial CAM expression was determined at 4 hours after reoxygenation. Each value represents mean ± SE of 3 experiments performed in triplicate. *P<0.05, **P<0.01, and ***P<0.001 compared with normoxia (control); #P<0.01 compared with A/R.
The enhanced A/R-induced neutrophil adhesion response mediated by T lymphocytes was completely blocked by anti-E-selectin. Collectively, these results support the contention that the T-lymphocyte-mediated enhancement of A/R-induced neutrophil–endothelial cell adhesion results from an increased E-selectin expression that is induced by TNF-α from activated T cells caused by postanoxic endothelial cell production of H$_2$O$_2$.

**Discussion**

It is well appreciated that the inflammatory response elicited by A/R is associated with enhanced neutrophil adhesion to endothelial cells. Although most analyses of leukocyte recruitment in posts ischemic tissues have focused on neutrophils, which appear to represent the ultimate circulating cell that mediates I/R-induced microvascular dysfunction, there is growing evidence that T lymphocytes also contribute to the pathogenesis of I/R injury. Indeed, we recently demonstrated a direct interaction of postanoxic endothelial cells with T lymphocytes at 8 hours after reoxygenation that is mediated by very late antigen-4/vascular cell adhesion molecule-1 and lymphocyte function—associated antigen-1/ICAM-1 interactions and involves a role for interleukin-8. These results indicate that T lymphocytes directly contribute to the A/R-induced inflammatory process by eliciting T-cell–endothelial cell interactions. The present study provides evidence in support of the hypothesis that T lymphocytes contribute indirectly to the A/R-induced inflammatory response by exacerbating neutrophil–endothelial cell adhesion via TNF-dependent induction of E-selectin.

Our data are consistent with a specific role for T lymphocytes, as evidenced by the finding that T cells, rather than B cells, account for essentially all the lymphocyte-mediated enhancement of A/R-induced neutrophil adhesion that was elicited by a mixed population of T and B lymphocytes. This observation agrees with in vivo studies that implicate T cells, but not B cells, in the neutrophil recruitment and microvascular dysfunction observed in the postischemic liver. Our use of cell culture inserts provides novel insights into the mechanisms that underlie the T-lymphocyte-mediated enhancement of A/R-induced neutrophil–endothelial cell adhesion. It is notable that a direct interaction between T lymphocytes and endothelial cells was not required for the enhanced inflammatory response; our data implicates T-lymphocyte–derived TNF-α as the soluble factor that exacerbates A/R-induced neutrophil adhesion. These results are consistent with in vivo studies demonstrating elevated plasma levels of TNF-α after I/R and an attenuating effect of TNF-α antibodies on I/R-induced leukocyte–endothelial cell adhesion.

The finding of the present study that media obtained from A/R-conditioned HUVECs significantly increased neutrophil adhesion to naïve HUVECs cocultured with T lymphocytes suggests that a soluble factor(s) elaborated from postanoxic endothelial cells leads to the activation of T lymphocytes. The
The attenuating effect of CAT, but not SOD or a PAF antagonist, on the T-lymphocyte-mediated enhancement of A/R-induced neutrophil–endothelial cell adhesion is consistent with a role for endothelial cell–derived H₂O₂ in this process. This suggestion was supported by the result that treatment of naive HUVECs directly with H₂O₂ mimicked the A/R-induced adhesion response. The observation that CAT also attenuates the exaggerated production of TNF-α by T lymphocytes incubated with conditioned media from postanoxic HUVECs further supports a role for H₂O₂ as the stimulus for T-cell activation in this model.

An interesting and potentially important observation in the present study is that T cells promote A/R-induced neutrophil–endothelial cell adhesion by specifically inducing the expression of E-selectin. Moreover, the finding that T cells caused the upregulation of E-selectin in the absence of direct interactions between T lymphocytes and endothelial cells (ie, by using cell inserts) suggests that a soluble mediator(s) from activated T lymphocytes stimulates endothelial cells to express E-selectin. The attenuating action of anti–TNF-α on E-selectin expression and enhanced neutrophil adhesion is consistent with a role for this proinflammatory cytokine in mediating the upregulation of the adhesion glycoprotein. The attenuating action of anti–E-selectin on T-lymphocyte–enhanced neutrophil adhesion to postanoxic endothelial cells is further evidence that E-selectin mediates this T-cell effect.

The observation that CAT attenuates the T-lymphocyte–mediated enhancement of A/R-induced neutrophil–endothelial cell adhesion strongly implicates HUVEC-derived H₂O₂ in the activation of T lymphocytes and the subsequent upregulation of E-selectin. Our findings also implicate TNF-α as the chemical link between T-cell activation and endothelial expression of E-selectin. The mechanism by which TNF-α promotes E-selectin expression on endothelial cells remains unclear. One possibility is that TNF-α activates the nuclear transcription factor, nuclear factor-κB (NFκB), which favors the transcriptional upregulation of E-selectin. We have previously demonstrated a role for both NFκB and activator protein-1 in A/R-induced transcription-dependent expression of E-selectin, which was initiated by a redox imbalance. The mechanism by which endothelial cell–derived H₂O₂ induces TNF-α release from activated T lymphocytes is also unclear. Previous studies have implicated H₂O₂-mediated redox signaling and NFκB activation in T-lymphocyte activation. Collectively, these observations suggest the potential involvement of oxidant-mediated NFκB signaling in T-lymphocyte activation and TNF-α release, which in turn induces E-selectin expression and neutrophil–endothelial cell adhesion (Figure 13).

In conclusion, the present study demonstrates that A/R initiates T-lymphocyte activation, which ultimately leads to an exaggerated neutrophil–endothelial cell adhesion. Our data are consistent with an early production and liberation of H₂O₂ by postanoxic endothelial cells, which in turn leads to the activation of T lymphocytes. The TNF-α that is released from the activated T cells specifically upregulates E-selectin on endothelial cells and promotes neutrophil–endothelial cell adhesion (Figure 13). This mechanism may provide a molecular basis for recent reports that describe a modulating influence of T lymphocytes in the microvascular dysfunction and parenchymal cell injury associated with I/R.

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References


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Materials and Methods

Materials. The following reagents were obtained from Sigma Chemicals Co. (St. Louis, MO): Histopaque 1077, catalase, and superoxide dismutase (SOD). Endothelial cell growth medium and bovine brain extract were purchased from Clonetics (San Diego, CA), while 1,11-dioctadecyl-13,3,31,31,3-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) was obtained from Biomedical Technologies, Inc. (Stoughten, MA). Fetal calf serum was obtained from Hyclone Laboratories Inc. (Logan, Utah). Mouse antihuman factor VIII was purchased from Calbiochem (San Diego, CA), while WEB2086 was obtained from Boehringer (Ingelheim, Germany). The monoclonal antibodies (mAb) used in this study were 8.4A6 (murine IgG1 Antihuman ICAM-1), PB1.3 (murine IgG1 Antihuman P-selectin), CL3 (murine IgG1 F(ab')2 Antihuman E-selectin), and Antihuman TNF-α (mouse IgG1, R&D Systems, Minneapolis, MN). 8.4A6, PB1.3 and CL3 were generously provided by Dr. Donald Anderson from Pharmacia-UpJohn Laboratories (Kalamazoo, MI).

Subjects. All procedures used to obtain human neutrophils and lymphocytes as well as human umbilical cords were approved by the Institutional Review Board for Human Research at the Louisiana State University Medical Center. Freshly discarded human umbilical cords were obtained from the delivery suite of Louisiana State University Medical Center. Each subject donating blood provided written consent and was compensated for participating in the study.

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by 0.25% collagenase treatment for 20 min at 37°C, as previously described\(^4\)\(^{13}\). The cells were grown in endothelial cell growth medium (EGM), supplemented with bovine brain extract. The cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO\(_2\) and were
expanded by brief trypsinization with 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.02% EDTA. Primary passage HUVEC were seeded onto fibronectin (25 µg/mL)-coated, 11 mm, 48-well tissue culture plates. Culture medium was replaced every second day. Cells were identified as endothelial cells by their cobblestone appearance at confluency, and positive labeling with acetylated low density lipoprotein labeled with Dil-Ac-LDL or mouse antihuman factor VIII. Passage 1 cultures were used for the studies.

**Isolation of neutrophils.** Human neutrophilic polymorphonuclear cells (PMNs) were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on Histopaque 1077. This procedure yields a PMN population that is 95% to 98% viable (by trypan blue exclusion) and 98% pure (by acetic acid-crystal violet staining).

**Isolation of lymphocytes.** Human peripheral blood mononuclear leukocytes were isolated from heparinized peripheral blood using gradient separation on Histopaque-1077, washed three times in Ca²⁺ and Mg²⁺-free PBS containing 5% fetal calf serum (FCS). Total lymphocytes (T- and B-lymphocytes) and the T-lymphocyte population were isolated by the method of Berney 14. To avoid cell activation during purification, a negative sorting strategy was employed. During separation of total lymphocytes, cells were incubated for 15 min on ice with anti-CD14 (Ancell, Bayport, MN) and anti-CD56 (Becton Dickinson, San Jose, CA) antibodies at 1 µg/2 x 10⁷ cells. During separation of T-lymphocytes, cells were incubated for 15 min on ice with anti-CD14, anti-CD56, and anti-CD19 (Southern Biotechnology Associates, Birmingham, AL) antibodies, at 1 µg/2 x 10⁷ cells. The cells were washed once, resuspended in 40 µL PBS / 10⁷ cells and incubated a further 15 min with goat anti-mouse IgG-magnetic beads (10 µL/10⁷ cells). The cells were washed once and
resuspended in 500 μL Ca²⁺ and Mg²⁺-free PBS containing 1% FCS. The suspension was then applied to a MACS column (Miltenyi Biotech, Auburn, CA), and the unlabeled cells were collected. The mononuclear cells obtained in the sort were lymphocytes without significant contamination by NK cells and monocyte. The T- lymphocyte cell sorts were without significant contamination by B cell, NK cell and monocytes.

A/R protocol. The in vitro model of anoxia-reoxygenation used in this study is similar to that previously reported ¹⁵, and has been used extensively in our laboratory ³. Confluent HUVEC monolayers were exposed to anoxia by incubating in a Plexiglas chamber that was continuously purged (1 L/min) with an anoxic gas mixture (93% N₂-5% CO₂-2% H₂). To ensure an oxygen-free environment, the gas mixture was passed through a catalytic deoxygenizer (Fisher Chemical, Medford, MA) before entry into the chamber. Chamber pO₂ was monitored during the entire experiment using an oxygen electrode (model OM-1, Microelectrodes, Londonderry, NH). In additional experiments, deoxygenation of the liquid layer covering the endothelial cells was confirmed using an oxygen microelectrode. The chamber and fluid phase pO₂ were directly related and reached a value of 0 mmHg within 5 min after initiation of gas flow. Temperature in the chamber was maintained at 37°C with a heating pad. After 60 min anoxia, reoxygenation was initiated by exposing the endothelial cells to room air for 4 h. Reoxygenation of endothelial monolayers was performed in the CO₂ incubator. Control endothelial cells were exposed to normoxia (21% O₂/5% CO₂/74% N₂) for the same duration of the experiment (normoxic controls). In a separate series of experiments, HUVEC monolayers were exposed to H₂O₂ (0.05 or 0.1 mmol/L) in the absence and presence of T-lymphocytes that were separated from the HUVEC monolayers by a 0.45 μm filter. After 45 min, T-lymphocytes were removed and neutrophil
adhesion assay was performed after 4 h of H2O2 exposure.

Adhesion assays. Isolated neutrophils were suspended in phosphate-buffered saline (PBS) and radiolabeled by incubating PMN (at a concentration of 2 x 10⁷ cells/mL) with 30 μCi Na⁵¹CrO₄ per mL neutrophil suspension at 37°C for 1 h. The cells were washed twice with ice-cold PBS (4°C), spun at 250 x g for 4 min to remove unincorporated radioactivity and resuspended in plasma-free Hanks' balanced salt solution (HBSS). Labeled neutrophils were added to HUVEC monolayers at a concentration of 1 x 10⁶ neutrophils/500 μL/well. Since the monolayers contained approximately 1 x 10⁵ cells /well, the resulting ratio of neutrophils to HUVEC was 10:1. In previous studies, we have optimized the use of this ratio of neutrophil to endothelial cell of 10:1 for our adhesion assay.⁴ After co-incubation (30 min) under static conditions, the supernatant was removed, and the monolayers were washed twice with HBSS. The remaining endothelial cells and adherent neutrophils were solubilized with 1N NaOH. The⁵¹Cr activity of the supernatant, washed fluid, and lysate were assessed in a gamma counter. The percent of added neutrophils that adhered to the HUVEC monolayers was quantified as follows:

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\frac{\text{Lysate (cpm)} \times 100}{\{\text{Supernatant (cpm)} + \text{Wash (cpm)} + \text{Lysate (cpm)}\}}
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Each adhesion assay was performed 4 h after reoxygenation. To assess the role of T- and B-lymphocytes or T-lymphocyte alone in the primary initiating event that promote neutrophil-endothelial cell interaction at 4 h reoxygenation, HUVEC monolayers exposed to 60 min anoxia were overlaid with suspensions of total lymphocytes or T-lymphocytes (T-cell to HUVEC ratio of 5:1) immediately after reoxygenation, and incubated for 45 min. In the current study, the ratio of T-lymphocytes to endothelial cells were selected on the basis that the proportion of
circulating T-cells is 50% that of circulating neutrophils. After co-incubation of lymphocytes with HUVECs, the cells were removed by two washes with HBSS. In some experiments, T-lymphocytes were separated from HUVEC monolayers by 0.45 μm millipore filters (Becton Dickinson, Franklin Lakes, NJ). In another series of experiments, HUVEC monolayers were exposed to 60 min anoxia and 45 min reoxygenation, and thereafter, the supernatants were collected, and added to naive HUVEC monolayers co-incubated with T-lymphocytes. After 45 min of co-incubation, T-lymphocytes were removed. These experiments will test the influence of supernatants from A/R-conditioned endothelial cells on neutrophil adhesion to naive HUVEC monolayers in the presence of T-lymphocytes. To assess whether superoxide or hydrogen peroxide (H$_2$O$_2$) play a role in the enhanced effect of A/R-induced neutrophil hyperadhesivity by T-lymphocytes, we treated monolayers with the respective antioxidant enzymes, superoxide dismutase (SOD; 1,000 U/mL), or catalase (1,000 U/mL) at concentrations that effectively scavenge the reactive oxygen metabolites. A role for platelet-activating factor (PAF) was determined using a blocking dose of PAF receptor antagonist (WEB 2086; 10 μmol/L). In previous studies, we and others have documented the utility and specificity of these inhibitors to attenuate ROS- or PAF-mediated neutrophil adhesion to endothelial cells. SOD, catalase, or PAF receptor antagonist were added to HUVEC prior to exposure to A/R. In experiments to investigate the contribution of TNF-α to the enhanced effect of A/R-induced neutrophil adherence to HUVEC by T-lymphocytes, T-cells on filter inserts were co-cultured with HUVEC monolayers in the presence of anti-TNF-α mAb (10 μg/mL). To test for a role of E-selectin on T-lymphocyte enhanced A/R-induced neutrophil adherence to HUVEC monolayers, T-cells were co-cultured with endothelial
cell monolayers in the presence of a blocking dose (20 µg/mL) of the monoclonal antibody (mAb) directed against E-selectin. The mAb was added to HUVEC monolayers 15 min before the adhesion assay.

**Endothelial cell adhesion molecule expression.** HUVECs were plated in 24 well tissue culture dishes. Primary antibodies (4 µg/mL) for either ICAM-1, P-selectin, or E-selectin in HBSS/PBS with 5% FCS were added to each well and incubated for 30 min at 37°C. The cells were washed and incubated for 30 min with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (IgG1 + IgG2a + IgG2b + IgG3, Southern Biotechnology Associates, Inc) diluted 1:5000 in HBSS/PBS with 5% FCS. The wells were then washed, and the binding of antibody was detected by the addition of 100 µL of 0.1 mg/mL 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co) with 0.003% H₂O₂. The reaction was stopped by the addition of 75 µL of 8N sulfuric acid. The samples were transferred to 96-well plates, and color development was read on a spectrometer (Titertek Multiskan MCC/340, ICN) at an OD of 450 nm after subtracting the background values in cells stained with a non-binding primary antibody and the second-step antibody.

**TNF-α assay.** To determine the levels of TNF-α, media were obtained from A/R-conditioned HUVEC monolayers without or with co-culture with T-lymphocytes on filter inserts, and in the absence or presence of catalase. The cytokine concentration was quantified using a commercially available human TNF-α ELISA kit (Endogen, Woburn, MA).

**Statistical analysis.** All values are expressed as mean ± SE. Data were analyzed by one-way ANOVA with Bonferroni corrections for multiple comparisons or Fisher’s PLSD.