Roles of tumor necrosis factor p55 and p75 receptors in TNF-α-induced vascular permeability

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Ferrero, Elisabetta, Maria Raffaella Zocchi, Elena Magni, Maria Carla Panzeri, Flavio Curnis, Claudio Rugarli, Maria Elena Ferrero, and Angelo Corti. Roles of tumor necrosis factor p55 and p75 receptors in TNF-induced vascular permeability. Am J Physiol Cell Physiol 281: C1173–C1179, 2001.—We have investigated the role of p55 and p75 tumor necrosis factor receptors 1 and 2 (TNFR1 and TNFR2, respectively) in TNF-induced alteration of endothelial permeability in vitro and in vivo. Stimulation of TNFR1 with an agonist antibody or a receptor-selective TNF mutein increased the flux of 125I-albumin through endothelial cell monolayers. An antagonist anti-TNFR1 antibody, but not antagonist anti-TNFR2 antibodies, blocked the activity of TNF in vitro. Stimulation of TNFR1, but not TNFR2, induced cytoskeletal reorganization associated with increased permeability. SB-203580, a p38 mitogen-activated protein kinase inhibitor, blocked TNFR1-induced cytoskeletal reorganization and permeability. A selective mouse TNFR1 agonist and human TNF, which binds to murine TNFR1, increased the leakage of trypan blue-albumin from liver vessels in mice. These results indicate that stimulation of TNFR1 is necessary and sufficient to increase endothelial permeability in vitro and in vivo. However, an antagonist anti-murine TNFR2 antibody partially inhibited the effect of murine TNF on liver vessels, suggesting that TNFR2 also plays a role in the regulation of TNF-induced vascular permeability in vivo.

TUMOR NECROSIS FACTOR-α (TNF-α) is an inflammatory cytokine that is produced by activated macrophages and T cells and in lower amounts by endothelial cells and other tissues (14). TNF is expressed as a 26-kDa transmembrane precursor from which a soluble 17-kDa polypeptide is released by proteolytic cleavage (19). Biological activities are induced upon interaction of TNF homotrimers with, and subsequent clustering of, two distinct cell surface receptors of 55- to 60-kDa and 75- to 80-kDa [tumor necrosis factor receptors 1 and 2 (TNFR1 and TNFR2), respectively] (39). Among the various cells that are affected by TNF during the inflammatory response, endothelial cells are a primary target. On these cells, TNF exerts multiple biological effects, including induction of leukocyte adhesion molecules (30, 32) and proinflammatory cytokines (18, 23) and fibrin deposition (24, 25) and modulation of nitric oxide production (11, 43).

Moreover, TNF induces alterations of endothelial cytoskeletal actin and formation of intercellular gaps with increased permeability to macromolecules (4, 17). Regulation of vascular permeability, together with induction of leukocyte adherence and procoagulant activity on the vessel surface, are critical events in the physiological response to several inflammatory/immunological stimuli and in a variety of pathological conditions, including endotoxic shock, systemic inflammatory response, and adult respiratory distress syndrome (36), as well as in the therapeutic and toxicological response to TNF infused at high doses in cancer patients (15). Studies aimed at elucidating the mechanisms of TNF-induced vascular permeability may be, therefore, of physiopathological, therapeutic, and toxicological interest.

In this study, we investigated the function of the two TNF receptors in TNF-induced alteration of the endothelial barrier function. To this aim, we have evaluated the effect of receptor-agonist/antagonist compounds (antibodies and TNF muteins selective for TNFR1 or TNFR2) on the permeability of human umbilical vein endothelial cell (HUVEC) monolayers. In addition, we investigated the activity of receptor-selective agonists on cytoskeletal reorganization in vitro and on liver vessel leakage in vivo in an animal model.

We show that stimulation of TNFR1 on endothelial cells is sufficient and necessary to trigger morphological changes that result in increased permeability to macromolecules in vitro. Moreover, we provide evidence to suggest that both TNFR1 and TNFR2 contribute to the TNF-induced leakage of solutes from liver vessels in mice.

MATERIALS AND METHODS

Chemicals and antibodies. Murine and human recombinant TNF (5 × 107 U/mg) were produced by cDNA expression.
in *Escherichia coli* (26, 27). The products were purified to homogeneity from *E. coli* crude extracts by ammonium sulfate precipitation, followed by hydrophobic interaction chromatography on phenyl-Sepharose, ion-exchange chromatography on DEAE-Sepharose, and gel filtration chromatography on Sephacryl-S-300 (Pharmacia-Uppjohn, Uppsala, Sweden). The protein content was measured using a commercial protein assay kit (Pierce, Rockford, IL). The bioactivity of TNF was estimated by standard cytotoxic assay using L-M mouse fibroblasts (American Type Culture Collection; CCL1.2) (7). Endotoxin content was <0.5 U/µg, as measured by the Lymulus Amoebocyte Lysate Pyrotest (Difco Laboratories, Detroit, MI). Human TNF muteins, including R32W-S86T, specific for TNFR1, and D143N-A145R, specific for TNFR2 (20), were kindly supplied by Dr. H. Loetscher (Roche, Basel, Switzerland). Monoclonal antibody (MAb) htr-9 (anti-human TNFR1) and utr-1 (mouse anti-human TNFR2) (5) were purchased from BMA Biomedicals, ( AUG, Switzerland). MAb 7H3 (mous anti-human TNFR1) and MAb 6G1 (rat anti-murine TNFR2) were described previously (6, 7). MAb V1q (rat anti-murine TNF) (8) was kindly supplied by Dr. D. Mannel (University of Regensburg, Regensburg, Germany). MAb 78 (mouse anti-human TNF) (1) was a gift from Dr. E. Barbanti (Pharmacia-Uppjohn, Milan, Italy). PD-98059 and SB-203580 were from Alexis Biochemicals (San Diego, CA).

**Cell isolation and culture.** Endothelial cells were isolated from human umbilical veins by collagenase treatment as described (12) and cultured in 1% gelatin-coated flasks (Falcon; Becton Dickinson, Bedford, MA) using endotoxin-free medium 199 (Sigma, St. Louis, MO) containing 20% heat-inactivated fetal calf serum and 1% bovine serum albumin (BSA; 1 mCi/ml; NEN, Boston, MA). The cells were washed again and blocked by an antagonist anti-TNFR1 antibody.

**RESULTS**

TNF-induced permeability of HUVEC monolayers is blocked by an antagonist anti-TNFR1 antibody. The expression of TNFR1 and TNFR2 on HUVEC at early passages was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later passages, each monolayer was first verified by fluorescence-activated cell sorter analysis. As expected, cells within four passages expressed both TNFRs (not shown). Because TNFR2 membrane expression could be lower at later
passages (31), only cells within four passages were used for subsequent studies.

The effect of TNF on the transit of radiolabeled albumin through confluent HUVEC monolayers was then investigated at various times using the double chamber permeability assay. The transendothelial \(^{125}\text{I-}\text{albumin}\) flux was significantly increased in the presence of 5 ng/ml of human TNF compared with controls without TNF (Fig. 1). This effect was prevented by MAb 7H3, an antagonist anti-TNFR1 (6), but not by utr-1, an antagonist anti-TNFR2 (5), or by a rabbit polyclonal anti-TNFR2 antiserum. This suggests that binding of TNF to TNFR1, but not to TNFR2, is necessary for mediating the TNF-induced permeability of HUVEC monolayers to \(^{125}\text{I-}\text{albumin}\). Of note, MAb 7H3 also decreased the spontaneous transit of \(^{125}\text{I-}\text{albumin}\) through unstimulated cell monolayers (Fig. 1, left).

**TNFR1-specific agonists increase the permeability of HUVEC monolayers.** The permeability of HUVEC monolayers to \(^{125}\text{I-}\text{albumin}\) was then measured in the presence of receptor agonists selective for TNFR1 or TNFR2, including 1) the anti-TNFR1 MAb htr-9, 2) a TNF mutein selective for TNFR1 (R32W-S86T), and 3) a mutein selective for TNFR2 (D143N-A145R). The agonistic activities of htr-9 and muteins have been demonstrated previously in several TNF-dependent assays (5, 10, 20, 42). As shown in Figs. 2 and 3A, MAb htr-9 and R32W-S86T mutein significantly increased the transit of \(^{125}\text{I-}\text{albumin}\) through HUVEC monolayers, whereas no significant effects were observed with the TNFR2 agonist (D143N-A145R). These findings, obtained with independent agonists of TNFR1, indicate that TNFR1 stimulation is sufficient to increase the permeability of HUVEC monolayers.

To test the requirements of TNFR1-initiated protein phosphorylation in mediating the vascular permeability increase, we studied the effects of various mitogen-activated protein (MAP) kinase inhibitors on HUVEC permeability after treatment with R32W-S86T. SB-203580, a p38 MAP kinase inhibitor, efficiently inhibited the TNFR1-induced permeability (Fig. 3B). In contrast, no inhibition of permeability was observed with PD-98059, a selective inhibitor of MAP kinase kinase (MEK).

**A TNF mutein selective for TNFR1 induces HUVEC cytoskeletal reorganization.** TNF-\(\alpha\) is known to promote changes in the size and shape of HUVECs by converting actin peripheral bundles into stress fibers (16). Cytoskeleton reorganization is accompanied by mutual cell retraction, resulting in formation of intercellular gaps. Because this phenomenon is believed to

![Fig. 2. Effect of an agonist anti-TNFR1 antibody on the permeability of HUVEC monolayers.](image)

![Fig. 3. Effect of receptor-specific TNF muteins on the permeability of HUVEC monolayers.](image)
correlate with an increase in permeability to macromolecules, the effect of TNF muteins on HUVEC cytoskeleton was then investigated by confocal microscopy analysis.

Human TNF and R32W-S86T, but not D143N-A145R, induced a marked actin reorganization (Fig. 4, A–D), indicating that TNFR1 stimulation is sufficient to trigger morphological changes in HUVECs. No effects were induced by D143N-A145R, even at fivefold higher concentration (not shown). The cytoskeletal reorganization of HUVECs was totally inhibited by the p38 MAP kinase inhibitor SB-203580 (Fig. 4E). In contrast, no inhibition of cytoskeletal reorganization was observed with MEK inhibitor PD-98059 (not shown). These results indicate that stimulation of TNFR1 alone is sufficient to trigger a cascade of events leading to cytoskeletal reorganization and that activation of the p38 MAP kinase pathway is necessary.

**Human TNF increases leakage from liver vessels in mice.** To assess whether stimulation of TNFR1 alone is sufficient to increase the vascular permeability in vivo, we investigated the activity of mouse and human TNF on the vascular leakage of trypan blue-albumin from liver vessels in an animal model. This system relies on the fact that mouse TNF can bind both TNFR1 and TNFR2, whereas human TNF can bind only the mu-
Table 1. Effect of mouse and human TNF on trypan blue-albumin retention in perfused mouse livers

<table>
<thead>
<tr>
<th>Effector</th>
<th>Dye Retention, 540 nm ± SE</th>
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<tbody>
<tr>
<td>None (saline)</td>
<td>327 ± 22</td>
</tr>
<tr>
<td>Human TNF</td>
<td>602 ± 51</td>
</tr>
<tr>
<td>Mouse TNF</td>
<td>872 ± 65</td>
</tr>
<tr>
<td>Human TNF + MAb 6G1</td>
<td>583 ± 36</td>
</tr>
<tr>
<td>Mouse TNF + MAb 6G1</td>
<td>572 ± 44</td>
</tr>
<tr>
<td>Human TNF + MAb 78</td>
<td>402 ± 25</td>
</tr>
<tr>
<td>Mouse TNF + MAb V1q</td>
<td>314 ± 29</td>
</tr>
<tr>
<td>MAb 6G1</td>
<td>379 ± 33</td>
</tr>
<tr>
<td>MAb 78</td>
<td>346 ± 29</td>
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<tr>
<td>MAb V1q</td>
<td>338 ± 38</td>
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Values are means ± SE. Dye retention assay was carried out as described in MATERIALS AND METHODS. Human or mouse tumor necrosis factor (TNF; 0.1 ng/g body wt) were mixed for 1 h at room temperature with monoclonal antibody (MAb) 78 (2 μg/ml) or MAb V1q (10 μg/ml) or MAb 6G1 (2 μg/ml), respectively, before injection. Means ± SE of 6 independent experiments.

Rine TNFR1 (34), thereby acting as a natural specific mutein in mice. Human or mouse TNF was injected intraperitoneally in BALB/c mice 20 min before liver perfusion with trypan blue-albumin. The leakage of dye was compared with that observed in animals treated with a physiological solution in place of TNF.

As shown in Table 1, pretreatment with either murine or human TNF induced dye retention within the liver. Both effects were neutralized by the relevant anti-TNF neutralizing antibodies (MAb V1q and MAb 78, respectively), confirming that the effects observed were related to TNF activity. Thus selective stimulation of TNFR1 was sufficient to decrease the barrier function of endothelial cells in vivo. However, an antagonist anti-murine TNFR2 antibody (6G1) partially inhibited murine TNF but not human TNF, suggesting that TNFR2 is also involved in the regulation of TNF-induced permeability in vivo.

**DISCUSSION**

Soluble TNF can increase the transit of macromolecules across vascular endothelium (4, 16, 17). To examine the role of the two TNFRs in mediating this phenomenon, we measured the passage of radiolabeled albumin through confluent HUVECs cultured on micro-porous polycarbonate membranes in the presence and absence of receptor agonist and -antagonist compounds.

We found that stimulation of TNFR1 with agonist compounds (MAb htr-9 and R32W-S86T mutein) can decrease the barrier function of endothelial cell monolayers, suggesting that TNFR1 plays a critical role in signaling TNF-induced endothelial permeability. This hypothesis is further strengthened by the observation that human TNF, a specific TNFR1 agonist in mice (34), increased the leakage of trypan blue-albumin complex in liver vessels. Thus stimulation of TNFR1 alone is sufficient for starting a cascade of events leading to increased permeability in vitro and in vivo. The finding that an antagonist TNFR1 MAb (7H3) can completely inhibit the effect of soluble TNF indicates that this receptor is not only sufficient but also necessary for activity. This MAb, directed to an epitope located within the fourth cysteine-rich domain of the receptor (6), was also able to decrease the spontaneous passage of radiolabeled albumin through the unstimulated cell monolayer. One possible explanation is that the spontaneous leakage is dependent, at least in part, on endogenous production of low levels of soluble or membrane TNF or on constitutive signaling by spontaneous clustering of TNFR1 in HUVEC monolayers.

In contrast, antagonist anti-TNFR2 antibodies were unable to affect the TNF-induced permeability in vitro. Moreover, a selective TNFR2 agonist (D143N-A145R mutein) failed to increase the permeability of endothelial monolayers. The unresponsiveness of endothelial cells to TNFR2 agonists/antagonists cannot be ascribed to the lack of expression of TNFR2, since we and others (21, 31) have found that HUVECs express both TNF receptors. It would appear, therefore, that the TNF-induced decrease of endothelial barrier function in vitro is under the dominant control of TNFR1.

It is generally believed that TNFR1 mediates most of the TNF effects on different cell targets (9, 10, 20, 28, 35). Endothelial permeability does not seem to be an exception, since we obtained evidence for a direct involvement of TNFR1 in endothelial barrier perturbation using different reagents, such as TNFR1 agonist and antagonist MAbs and TNF muteins. Although our data seem to exclude the possibility that TNFR2 signaling alone mediates the TNF-induced permeability of HUVECs, we cannot rule out the possibility that TNFR2 can indirectly regulate the TNFR1 signaling in vivo. Indeed, in vivo administration of an antagonist anti-TNFR2 antibody partially inhibited murine TNF, but not human TNF, hinting at a functional role of TNFR2 in the regulation of vessel permeability. It has been proposed that TNFR2, besides playing a direct role in some cellular responses triggered by soluble TNF (22, 34, 40), can also play accessory roles by increasing the local concentration of soluble TNF and rapid “ligand passing” to TNFR1 (2, 33) or by cooperative signaling (41). Ligand-induced formation of TNFR1 and TNFR2 heterocomplexes have also been demonstrated (29). It is possible that our antagonist anti-TNFR2 antibody blocked these accessory roles of TNFR2. Moreover, given the complexity of the vascular structure and the permeability process, we cannot exclude the possibility that other TNFR2-positive cells participate to regulate the vascular permeability in vivo.

We have previously reported that the effect of TNF on endothelial cell permeability is mediated, at least in part, by the destabilization of cytoskeletal/platelet endothelial cell adhesion molecule-1 (PECAM-1) connections, through increased PECAM-1 phosphorylation, and that this event, together with phosphorylation of other junctional molecules, is also critically involved in TNF-induced vascular permeability (13). Interestingly, we observed that p38 MAP kinase activation is necessary for TNFR1-induced cytoskeletal reorganization and permeability of HUVECs, since SB-203580, an inhibitor of this

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enzyme, completely prevented both HUVEC cytoskeletal reorganization and permeability triggered by TNFR1 in vitro.

Our finding that a TNFR1 agonist is sufficient to induce actin cytoskeletal reorganization suggests that TNFR1 is primarily involved in the dynamic regulation of the interactions between these cytoskeletal/junctional molecules. Considering that regulation of other adhesive molecules, such as intracellular adhesion molecule-1, E-selectin, vascular cell adhesion molecule-1, and CD44 are also under the dominant control of TNFR1 (21), it would appear that this receptor is primarily involved in the regulation of shape and adhesive properties of endothelial cells during the inflammatory response.

The effects triggered by TNFR1 on vascular permeability may account for some of the physiopathological effects of TNF during the acute phase response and other conditions associated with soluble TNF overproduction, such as endotoxic shock, systemic inflammatory response, and adult respiratory distress syndromes (36). Moreover, given that TNF is used as a drug for cancer therapy, the increase of endothelial cell permeability mediated by TNFR1 may also account for some of the toxic effects observed after regional or systemic administration of TNF-α (15) or TNFR1-specific muteins (37, 38).

In conclusion, we have shown that the capability of soluble TNF to perturb the barrier function of endothelium is under the dominant control of TNFR1 and that TNFR2 may play an accessory role in vivo. This notion may help in understanding the complex effects of this cytokine under pathophysiologcal conditions and in the development of specific pharmacological treatments aimed at reducing excessive increase in vascular permeability.

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