Both Tumor Necrosis Factor Receptors, TNFR60 and TNFR80, Are Involved in Signaling Endothelial Tissue Factor Expression by Juxtacrine Tumor Necrosis Factor α

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We have investigated the role of the two distinct tumor necrosis factor (TNF) receptors (TNFR60 and TNFR80) in endothelial cell activation employing an in vitro model of tumor necrosis factor α (TNF-α)-dependent tissue factor production of human umbilical vein endothelial cells (HUVECs). In this model, tissue factor is produced either on addition of exogenous TNF-α, or by induction of endogenous TNF-α via adhesion molecule-linked signal pathways. Under both conditions, tissue factor expression could be partially blocked by antagonistic antibodies against either TNFR60 or TNFR80 and was fully inhibited by simultaneous application of both antibodies. Selective inhibitors of either TNFR60 or TNFR80-induced signal pathways inhibited tissue factor expression, and selective triggering of either of the two TNF receptors by agonistic antibodies induced this response in HUVECs. Furthermore, a coculture system of HUVECs and Chinese hamster ovary transfectants expressing a noncleavable, exclusively membrane-bound form of TNF-α resulted in a potent activation of HUVECs with synergistic action of both TNF receptors. Together, these data underline the importance of juxtacrine pathways in endothelial cell activation of procoagulant functions and show that membrane TNF-α and both TNF types play a critical role.

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MATERIALS AND METHODS

Reagents. Recombinant human interferon γ (Dr K. Thomae GmbH, Biberach, Germany) and recombinant human TNF-α (Genentech, San Francisco, CA) were used at a final concentration of 50 ng/mL. Pargyline and nordihydroguaiaretic acid (NDGA) were purchased from Sigma Chemical Co (Deisenhofen, Germany) and were used at final concentrations of 250 and 3 μg/mL, respectively. D609 (Kamiya Biomedical Company, Thousand Oaks, CA) was diluted to a final concentration of 40 μg/mL. The generation of the monoclonal antibody (MoAb) H938, a TNFR80 specific antagonist,21 the rabbit antihuman TNFR80 serum M80,22 and the TNFR80-specific agonistic MoAb hr-1213 have been described previously. The purified IgG fraction from M80 serum was used for stimulation experiments. For TNFR80 inhibition studies, Fab fragments, generated from the IgG fraction of M80 serum were used as selective antagonists, as described.22 MoAb to E-selectin (ELAM, IgG2, mouse), as well as a MoAb specific for ICAM-1 (IgG1, mouse) were provided by Bender (Vienna, Austria). All antibodies were devoid of preservatives and were used at a final concentration of 50 μg/mL, except for cytofluorometric analysis.

Isolation and culture of endothelial cells. Endothelial cells from HUVECs were isolated according to the method of Jaffe et al24 and propagated in culture in medium M199 supplemented with CLEX (5% wt/vol, Interchem, München, Germany), heat inactivated fetal calf serum (5% wt/vol) and human serum (5% wt/vol) until they reached confluence. The cells were detached from the culture dish by collagenase treatment (1 mg/mL in phosphate-buffered saline expression in primary HUVEC cultures already declines at very early passages, (2) other studies with distinct cell types, showing a genuine, TNFR60 independent signal capacity of TNFR80 on appropriate activation,16,18 and (3) mouse tumor models suggesting involvement of TNFR80 and the host endothelium in induction of tumor necrosis.1,20 We scrutinized the functional role of TNFR80 in endothelial cell activation and analyzed potential mechanisms of cooperation with TNFR60. To this end, we have used second passage HUVECs in a model of in vitro tissue factor induction by endogenously induced or exogenously added, soluble or membrane bound TNFα. By using receptor subtype specific agonistic and antagonistic antibodies, as well as selective inhibitors of TNFR60 and TNFR80-induced signal pathways, we show that both TNF receptors induce signal pathways in HUVECs that synergistically interact to produce tissue factor.

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[LB], 2 minutes, 37°C), washed in M199 plus supplements, and then seeded on four-well plates (Nunclon, Nunc, Denmark) coated with extracellular matrix derived from bovine corneal endothelial cells. For assays, cells of the second passage were grown to confluence for 2 to 3 days in medium M199 plus supplements. Each well (diameter = 16 mm) contained approximately 1 × 10⁶ HUVECs.

Coculture of HUVECs and membrane TNF expressing transfectants. Stable CHO transfectants expressing a noncleavable, membrane-bound form of TNF-α (clone B2.20) have been constructed as described by Perez et al.⁵ TNF-α membrane expression and the complete lack of soluble, bioactive TNF of clone B2.20 was verified by fluorescence-activated cell sorter (FACS) analysis and standard TNF-α bioassays, respectively, and is described in detail elsewhere (Grell et al, submitted). For the experiments described herein, B2.20 and control CHO cells, respectively, were added to the endothelial cells at 2 × 10⁵ cells per well.

Antibody staining and cytofluorometric analysis. HUVECs were washed and detached from the extracellular matrix by collagenase treatment as described above. All MoAbs (H398 and isotype controls) were used at a final concentration of 5 µg/mL. M80 serum was used as a 1:100 final dilution. The antibodies were incubated at 4°C for 45 minutes in PBS containing 0.2% bovine serum albumin (BSA). The cells were washed with PBS/BSA and stained with a fluorescein isothiocyanate-coujugated F(ab)₂ goat antiserum and goat antirabbit IgG, respectively, for 45 minutes at 4°C. The cells were washed again and the pellets were resuspended in PBS/BSA. Control antibodies of the corresponding isotype and preimmune serum were used to determine nonspecific binding. Cytofluorometric analysis was performed with an Epics CS (Coulter, Hialeah, FL) at a wavelength of 488 nm and a Laser setting of 250 mW.

Antibody cross-linking of E-selectin and ICAM-1 and TNF-α production of HUVECs. Anti-E-selectin (ELAM) and anti-ICAM-1 were incubated with HUVECs, preactivated by interferon (IFN)γ treatment (50 ng/mL, 6 hours, 37°C), and washed extensively before further treatment with PBS as described. Antibody incubations were performed at 4°C for 1 hour. For antibody cross-linking, F(ab)₂ fragments of a goat antiserum (IgG) were added at a final concentration of 500 µg/mL after washing the cultures with PBS. The F(ab)₂ fragments were incubated for 1 hour at 4°C, as described above. After washing with PBS, the cells were maintained in M199 (plus supplements) at 37°C for 12 hours. At that time, TNF-α production of E-selectin/ICAM-1-stimulated HUVECs was determined in culture supernatants by a human TNF-α-specific enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Germany) according to the manufacturer’s instructions.

Blockade of TNFRs by antagonistic antibodies. After cross-linking of E-selectin and ICAM-1 as described above, HUVECs were washed with PBS and incubated at 4°C for 45 minutes with antibodies specific for TNFR60 (H398) and/or TNFR80 (M80 Fab fragments). The cells were washed with PBS and further incubated in 0.5 mL medium M199 (plus supplements) at 37°C for 12 hours. For blockade of membrane TNF-α-induced signal transduction, HUVECs were incubated, before addition of B2.20 cells to the cultures, in accordance with the reagents above.

Stimulation of TNFRs with agonistic antibodies. Endothelial cells were washed with PBS and separately incubated with agonistic antibodies against TNFR60 (50 µg/mL, h-tr) or TNFR80 (50 µg/mL, M80 IgG fraction), at 4°C for 45 minutes. After washing with PBS, 0.5 mL medium M199 (plus supplements) were added and the cells were incubated at 37°C for 12 hours.

Inhibition of TNF-induced signal pathways. For blockade of the TNF signal pathways, the inhibitors pargyline (250 µg/mL), NDGA (3 µg/mL) and D609 (40 µg/mL) were added to HUVECs after cross-linking of E-selectin and ICAM-1, being present during the 12-hour incubation period.

Tissue factor activity. The surface coagulant activity of the HUVEC monolayers was measured in a two-stage thrombin formation assay in human whole blood. A total of 0.6 mL of freshly drawn citrated human blood was recalcified and immediately added to the HUVECs. The four-well plate was shaken gently at 37°C. A total of 10 µL samples were taken at 1-minute intervals, for 20 minutes. The samples were diluted 1:400 in reaction buffer (TRIS-HCl 50 mmol/L, NaCl 100 mmol/L, EDTA 20 mmol/L, BSA 0.5 g/L, pH 7.9) containing 200 µmol/L chromogenic substrate S2238 (Kabi Vitrum, Malmö, Sweden). The reaction mixture was kept on ice for the duration of the experiment, then cellular components were removed by centrifugation. The chromogenic reaction was started by rapidly warming to 37°C. Absorption at 405 nm was measured at two time points (5 minutes and 10 minutes after the start of the warming) and optical density (OD) was determined. The amount of functionally active thrombin generated was calculated from a standard curve derived from purified human thrombin (kindly provided by Professor H.C. Hemker, Maastricht, Netherlands). Next, a dose response curve of recombinant human tissue factor (kindly provided by Professor W. Konigsberg, Yale University, New Haven, CT) was prepared. We determined the time delay (in minutes) until the rate of thrombin formation exceeded 30 nmol/L as a parameter to convert the onset of thrombin formation to tissue factor concentration. The same method was used to determine the amount of tissue factor on the surface of endothelial cells.

RESULTS

Both TNF receptors are involved in endothelial cell tissue factor production. To investigate the role of both TNF receptors in TNF-α signaling of tissue factor production, membrane expression of TNFR60 and TNFR80 on HUVECs from second passage in vitro culture was verified by FACS analysis. A typical result is shown in Fig 1, indicating that both TNFRs were expressed at approximately equal levels in these cells, with 63% and 68% of HUVECs staining at above background levels with TNFR60 and TNFR80 specific antibodies, respectively.

In the first set of experiments, tissue factor production was initiated by simultaneous cross-linking of E-selectin and ICAM-1, previously shown to stimulate endogenous TNF-α, which subsequently induces tissue factor expression in endothelial cells. In the experiments depicted in Fig 2A, a tissue factor production of 110 ± 15 pg/well was noted, whereas, in the absence of cross-linking adhesion molecule-specific antibodies, no tissue factor production was observed. When antagonistic antibodies against TNFR60 and TNFR80 were separately added to preactivated HUVECs directly after cross-linking of E-selectin and ICAM-1, the expression of functional tissue factor activity decreased to 50 ± 21 pg/well and 60 ± 17 pg/well, respectively (Fig 2A). Simultaneous blocking of both TNFRs reduced the amount of functionally active tissue factor to 30 ± 16 pg/well (Fig 2A). Independently, involvement of TNFR60 and TNFR80 in tissue factor induction was confirmed by stimulation with recombinant TNF-α (50 ng/mL), instead of endogenously produced TNF-α. Again, tissue factor activity (145 ± 18 pg/well) could be inhibited, both by antibodies against TNFR60 (50 ± 11 pg/well) and by antibodies against TNFR80 (85 ± 16 pg/well) (Fig 2B). Simultaneous blockade of both
Signal transduction inhibitors block E-selectin/ICAM-1 induced tissue factor production at the level of TNF receptor signaling. The above results indicated an involvement of both TNF receptors in induction of tissue factor gene expression. It was now of interest to determine the role of each of the TNF receptors in this response, in particular whether TNFR80 has an active, TNFR60 independent signaling function or is only involved as a ligand passing receptor. To this end, we used inhibitors previously shown to selectively interfere with TNF-α-induced intracellular signal pathways. Among several inhibitors shown to affect TNF-α signaling, two were of special interest because they allowed to discriminate between TNFR60- and TNFR80-induced signal pathways in a model of TNF-α-mediated tumor cell apoptosis: NDGA, known as an inhibitor of lipoxygenases and mono-oxygenases, was found to interfere with TNFR80 signaling, whereas Pargyline, a mono-amino-oxidase inhibitor, was shown to selectively affect TNFR60 induced cell death.

HUVECs were activated by E-selectin/ICAM-1-crosslinking, and cells were cultured in the absence or presence of inhibitors for 12 hours before tissue factor expression was determined. In parallel to tissue factor activity, TNF levels in the culture supernatants were measured by ELISA to ensure production of endogenous TNF-α (236 pg/mL) by E-selectin/ICAM-1 cross-linking (Fig 1). The presence of D609, a phosphatidylcholine-specific phospholipase C reagent, known as an effective inhibitor of TNF responsiveness in several, but not all, cell types, resulted in a strong inhibition of tissue factor expression (Fig 1). Likewise, the separate addition of the selective inhibitors of TNFR60 and TNFR80 signal pathways, pargyline and NDGA, in each case led to a reduction of tissue factor expression from 180 ± 16 pg/well to 40 ± 11 and 38 ± 12 pg/well, respectively (Fig 1). Equal TNF-α levels in all E-selectin/ICAM-1-activated HUVEC cultures verified that the inhibitors used were, indeed, selective for TNF-α-induced signal pathways and did not inhibit TNF production.
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Fig 3. E-selectin/ICAM-1–induced, TNF-dependent stimulation of tissue factor synthesis involves both TNFR60 and TNFR80 signal pathways. E-selectin/ICAM-1–cross-linking–induced tissue factor production in the absence or presence of various TNF-α signal transduction inhibitors. HUVECs were treated on antibody cross-linking with D609 (40 μg/mL), pargyline (250 μg/mL), or NDGA (3 μg/mL), and TNF-α, as well as tissue factor synthesis, was determined after a 12-hour treatment as described in Materials and Methods. Any direct influence of these compounds on TNF-α–independent tissue factor synthesis was excluded in control experiments of LPS-induced tissue factor production of HUVECs, which was found to be unaffected by all three substances (data not shown).

not interfere with adhesion molecule–induced signals leading to endogenous TNF-α production in HUVECs (Fig 3). These data suggest that both TNFR are capable of signal transduction in endothelial cells and that the pathways activated by TNFR60 and TNFR80, respectively, are distinct.

TNFR80 independently induces tissue factor production and synergizes with TNFR60 at the level of signal transduction. Direct experimental proof of an active signaling, rather than a supportive, ligand passing function of TNFR80 in HUVEC tissue factor production was obtained in two independent approaches. First, HUVECs were treated with agonistic antibodies htr-1 and M80, specific for TNFR60 and TNFR80, respectively. With each of these agonistic antibodies, a selective activation of HUVECs could be shown and costimulation of both TNFRs by agonistic antibodies resulted in a further increase in tissue factor production (98 ± 12 pg/well, Fig 4). Thus, from antibody mediated activation of each of the TNFRs, their signal capability in principle can be deduced. In quantitative terms, however, receptor activation by antibodies depends on inherent features of these individual reagents and cannot be directly compared with each other or to the natural ligand.

Therefore, in an approach to show an active signal capacity of each of the two TNFRs induced by natural ligand under conditions that rule out a function of TNFR80 as a ligand passing receptor, we used the transmembrane form of TNF as a stimulus for tissue factor production of HUVECs. Stable CHO transfectants (B2.20) have been established expressing a mutated form of TNF-α, in which normal proteolytic processing to the soluble 17-kD form is abrogated by internal deletion of 12 amino acids (nos. 1 through 12 of the mature protein). Similar constructs have previously been shown to possess bioactivity. B2.20 cells express high levels of TNF in the cell membrane, as shown by immunofluorescence microscopy, but do not release bioactive TNF into the culture supernatant (Grell et al, submitted). Stimulation of HUVECs with B2.20 cells induced large amounts of tissue factor, which rose from 2 ± 3 pg/well to 290 ± 15 pg/well (Fig 5), whereas coincubation of HUVECs with untransfected CHO cells did not induce tissue factor expression (5 ± 5 pg/well, Fig 5). The membrane TNF-α–induced tissue factor activity could be strongly inhibited by antibodies against TNFR60 (40 ± 7 pg/well) and TNFR80 (70 ± 12 pg/well). Simultaneous blockade of both TNFRs inhibited tissue factor synthesis close to background levels (20 ± 15 pg/well).
cell activation, including tissue factor production, via recent data from TNFR80 knock-out mice that exerted a finding can be deduced from the above-mentioned mouse one considers the role of the tumor vasculature critical in freshly isolated or earlier passage HUVECs (Fig 1840 SCHMID ET AL). Moreover, an in vivo function, eg, in lipopolysaccharide from TNFR80 (ligand passing model). Meanwhile, an in-duction of TNFR80 in many TNF-α responses was first evident from in vitro antibody inhibition studies and later in vivo studies with receptor-gene knock-out animals fully confirmed these predictions. A physiologic function for TNFR80 was less apparent until it was shown that there is a reduced efficacy of human as compared with mouse TNF-α in mouse tumor models that could be related to the fact that human TNF-α does not bind to the murine TNFR80. It was initially suggested that TNFR80 only functions to increase local concentrations of TNF-α at the cell membrane in the vicinity of TNFR60 because of a high off-rate of soluble TNF-α from TNFR80 (ligand passing model). Meanwhile, an independent signal capacity of TNFR80 has been clearly established and an in vivo function, eg, in lipopolysaccharide (LPS)-induced shock syndrome and tissue necrosis, is apparent from receptor-knock-out mice. The data presented here now show that TNFR80 also plays an active signaling role, independent of TNFR60, in tissue factor induction in endothelial cells. The potential physiological significance of this finding can be deduced from the above-mentioned mouse tumor models, suggesting an involvement of TNFR80, if one considers the role of the tumor vasculature critical in the initiation of necrosis, by induction of intravascular coagulation. This view of TNFR80 function is fully supported by recent data from TNFR80 knock-out mice that exerted a strongly reduced sensitivity to TNF-mediated tissue necrosis.

Our results are at variance with those obtained in a similar model by Paleolog et al., who could not show endothelial cell activation, including tissue factor production, via TNFR80 by itself. Two, mutually exclusive explanations might be considered to account for these discrepant findings: first, these authors used later passage cells, which, in agreement with our own observations, already have a reduced TNFR80 membrane expression as compared with freshly isolated or earlier passage HUVECS (Fig 1). Second, the functional activities of the antibodies used here (polyclonal IgG, recognizing multiple TNFR80 epitopes) and in their study (MoAb) are clearly different. It is our own experience that agonistic antibodies, used as those of others, that the agonistic activity of TNFR-specific MoAbs can differ considerably in different experimental models. In addition to receptor activation by agonistic antibodies, we have presented here two other independent lines of evidence for an active signaling of TNFR80 in HUVECs and a cooperation with TNFR60 at an intracellular signal pathway level (1) inhibition of TNF-α–induced tissue factor production by NDGA, a substance that has been previously shown to selectively interfere with TNFR80 and not TNFR60 signaling in a model of tumor cell cytotoxicity involving both TNFR types; (2) induction of tissue factor production by a nonsecretable, membrane-bound TNF-α, which formally rules out ligand passing as a mechanism of TNFR80 function, and inhibition of this response by TNFR80-specific antagonistic antibodies. Of note is that on activation with membrane bound TNFα, tissue factor production was particularly high and each of the receptor-specific, antagonistic antibodies caused a marked inhibition of tissue factor production. These data strongly support the view that, in HUVECs, synergistic signal pathways of the two TNFR are operative. Although further studies are required to elucidate in detail the molecular nature of the pathways used by each of the TNFRs in endothelial cells, both the antibody inhibition studies as well as those employing inhibitors of signal pathways suggest that for optimal tissue factor production by HUVECs, a cooperation of both TNFRs at a signal pathway level is necessary.

Our observation that membrane TNF-α is a very efficient module for induction of tissue factor gene expression points to the importance of cell-cell interactions and juxtacrine mechanisms for regulating endothelial functions in a local environment, such as the microvessels of a vascularized tumor or atherosclerotic lesions. We have previously shown that leukocyte adhesion to HUVECs induces endothelial tissue factor via endogenous TNF-α gene expression, which is initiated by a combined activation of ICAM-1 and E-selectin induced signal pathways. All cells capable of TNF-α secretion, including endothelial cells, produce TNF-α via the membrane-expressed 26-kD precursor. Accordingly, it is conceivable that a juxtacrine positive-feedback loop is initiated on leukocyte adhesion to the endothelium promoting and expanding the procoagulant state to neighboring endothelial cells by a mutual interaction of membrane TNF-α/TNFReceptors and stimulation of tissue factor synthesis.

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Both tumor necrosis factor receptors, TNFR60 and TNFR80, are involved in signaling endothelial tissue factor expression by juxtacrine tumor necrosis factor alpha

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