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Synergistic induction of endothelial tissue factor by tumor necrosis factor and vascular endothelial growth factor: functional analysis of the tumor necrosis factor receptors

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Abstract Tissue factor expression on the surface of endothelial cells can be induced by tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) in a synergistic manner. We have investigated the role of the two different TNF receptors for this synergy. Firstly, stimulation of the 60 kDa TNF receptor (TNFR60) by a mutant of TNF specific for TNFR60 induced responses comparable to wild-type TNF. In contrast, stimulation of TNFR80 by a TNFR80-specific TNF mutein did not result in enhancement of tissue factor expression even in the presence of a suboptimal TNFR60 triggering. Secondly, we tested neutralizing TNF receptor antibodies for inhibition of tissue factor synthesis induced by VEGF and TNF. A TNFR60-specific antibody inhibited tissue factor production over a broad range of TNF concentrations, indicating an essential role of TNFR60 in the TNF/VEGF synergy. In contrast, blocking of TNF binding to TNFR80 strongly inhibited TNF-induced tissue factor expression at low, but less pronounced at high, TNF concentrations. In conclusion, these data are in agreement with a model in which TNFR80 participates in the synergy between VEGF and low concentrations of soluble TNF by passing the ligand to the signalling TNFR60.

Key words: Receptor; Tumor necrosis factor; Vascular endothelial growth factor; Synergistic induction; Tissue factor

1. Introduction

TNF is a pleiotropic cytokine showing bioactivity on a variety of different cell types, a phenomenon which may be explained in part by the existence of two functionally distinct TNF receptors, designated TNFR60 and TNFR80. By means of selective agonistic/antagonistic TNF receptor antibodies [1] and mutants of TNF [2,3], specific for either one of the TNF receptors, the association of biological activities with the individual TNF receptors had been analyzed in vitro and in vivo. The cellular effects of TNF in vitro such as cytotoxicity towards tumor cells [1], stimulation of fibroblast growth [4], tissue factor induction in endothelial cells [5,6] and expression of endothelial cell adhesion proteins [7] are reportedly associated with signalling by the TNFR60 receptor. In contrast, GM-CSF secretion and thymocyte proliferation emerged to be mediated by the TNFR80 receptor [1,8]. In further in vivo studies, a TNF mutant specific for TNFR60 was shown to produce toxicity in baboons [9]. Furthermore, the essential role of the TNFR60 in TNF-induced host response (leukocyte organ infiltration) and lethal septic shock was confirmed by studies employing mouse mutants deficient in the TNFR60 [10–12], but TNFR80-deficient mice also appeared to be less sensitive to TNF-mediated lethality [13]. It has been proposed that the TNFR80 may participate in TNFR60 action by raising the actual concentration of TNF in proximity of the TNFR60. This ligand passing mechanism [14] should be most pronounced at low concentrations of TNF due to the lower K_d of the TNFR60 compared to the TNFR80.

Although TNF was discovered and isolated by its ability to induce hemorrhagic necrosis in transplantable methylcholanthrene (meth A)-induced fibrosarcomas of mice, the mechanism underlying this induction of tumor necrosis had not been intensively studied. The ability of TNF to exert toxic effects against a number of tumor cell lines in vitro was quoted in favor of a direct anti-tumor effect in vivo. As late as 20 years after the discovery of TNF as a tumor necrosis inducing factor, involvement of the tumor vasculature became obvious. Starting from studies showing that TNF does not affect unvascularized and intraperitoneally growing meth A sarcomas, intravasal fibrin deposition after systemic TNF treatment and inhibition of the antitumoral effect of TNF by anticoagulants were demonstrated [15,16]. In order to explain the exclusive tumor localization of these events, it was proposed that secreted tumor factors could enhance the TNF-mediated induction of endothelial tissue factor [15,17–19]. One of these cytokines, the vascular endothelial growth factor/vascular permeability factor (VEGF) can induce tissue factor production on cultured human endothelial cells. This production is supported synergistically by TNF concentrations as low as 5 pM [18]. As tissue factor is considered the major initiator of coagulation in vivo [20], this synergistic VEGF–TNF interaction may be an important mechanism in TNF-induced tumor necrosis [21].

Induction of tumor necrosis by systemic administration of TNF in mice is much more efficient with murine TNF than with human TNF [22]. Since human TNF does not bind to murine TNFR80 but to murine TNFR60, TNFR80 appears to be involved in the tumor necrosis induction by TNF. In addition, TNFR80 has also been shown to be essential for another TNF-induced necrosis, namely skin necrosis [23]. Therefore, it seems to be reasonable to expect that any in vitro candidate mechanism for tumor necrosis induction in vivo should be dependent on TNFR80. In this study we de-

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Abbreviations: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; VEGF, vascular endothelial growth factor

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scribe the results of experiments with receptor-selective TNF mutants and antagonistic antibodies directed against the two TNF receptors. These agents were analyzed for their ability to induce or inhibit the expression of tissue factor on endothelial cells in the presence of VEGF. While a TNFR60-specific TNF mutant was superior to TNFR80-specific TNF mutants for the induction of tissue factor, antagonistic antibodies to the TNFR80 were as effective as TNFR60 antagonistic antibodies in inhibiting tissue factor production by low concentrations of TNF. Therefore, a ligand passing mechanism is considered for the involvement of the TNFR80 in the TNF/VEGF synergy.

2. Experimental procedures

2.1. Materials

Chemicals were purchased from Sigma (Munich, Germany) if not listed otherwise. Media and usual culture reagents were obtained from GIBCO (Eggenstein, Germany). The TNFR80-specific polyclonal rabbit antiserum M80 and the production of antagonistic Fab fragments were produced as recently described [24]. Human umbilical cords were kindly donated from hospitals in the 'Wetterau'. Citrated pooled plasma was obtained from volunteers.

2.2. Cell culture and assays

Human umbilical vein endothelial cells (HUVEC) were prepared by the method of Jaffe [25] as modified by Thornton et al. [26]. HUVECs were cultured in MDCB131 medium supplemented with 10 mM HEPES (pH = 7.4), 10% FCS (PAA, Linz, Austria), 100 μg/ml endothelial cell growth factor (ccpro, Neustadt, Germany), heparin (20 μg/ml), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 2.5 μg/ml fungizone. Experiments were carried out within 48 h of the cells achieving confluence. Expression of tissue factor in endothelial cells was assessed by incubating cultures with TNF and/or purified recombinant VEGF in MDCB131 containing 10 mM HEPES (pH = 7.4), 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, in the presence/absence of antibodies. For studies with neutralizing antibodies, cells were submitted to a 15 min incubation period before addition of the cytokines. Cells were incubated for 6 h at 37°C. Assays were carried out with whole cells obtained in suspension following scraping and tissue factor activity equivalents were determined as previously described [18].

2.3. Cytofluorometric analysis and binding studies with [^{125}I]TNF

HUVECs were washed and detached with 5 mM EDTA in HBSS/25 mM HEPES. Incubation time with trypsin was not allowed to exceed 3 min in total. Cells were incubated with primary unconjugated antibodies for 30 min at 4°C and 5 μg/ml as final concentration, washed twice with FACS buffer (PBS supplemented with 1% BSA and 0.1% NaN_3) and then incubated with FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. After washing twice, the cells were either analyzed immediately or fixed in 1% paraformaldehyde in PBS and stored at 4°C for later analysis. Flow cytometry analysis was performed on a FACStar (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA). TNF was labelled as described recently [6]. For competition binding studies cells were preincubated in triplicates with the specific TNFR antibodies H398 and utr-1 (30 μg/ml each) for 90 min followed by an incubation period of 2 h with 20 ng/ml ^{125}I-labeled TNF. Cells were washed twice and radioactivity was determined in a gamma-counter. All steps were carried out on ice.

Table 1
Binding competition of TNFR-specific antibodies with [^{125}I]TNF to HUVECs (P2)

Addition	Mean ^{25}J cpm ± SD	% Competition
None	3987 ± 58	–
Unlabeled TNF	1954 ± 50	100
H398	2905 ± 73	53
utr-1	3320 ± 97	33
H398+utr-1	2046 ± 103	95

HUVECs were preincubated with/without either anti-TNFR80 (utr-1), anti-TNFR60 (H398), both antibodies or an excess of unlabeled TNF before incubation with 20 ng/ml [^{125}]TNF.

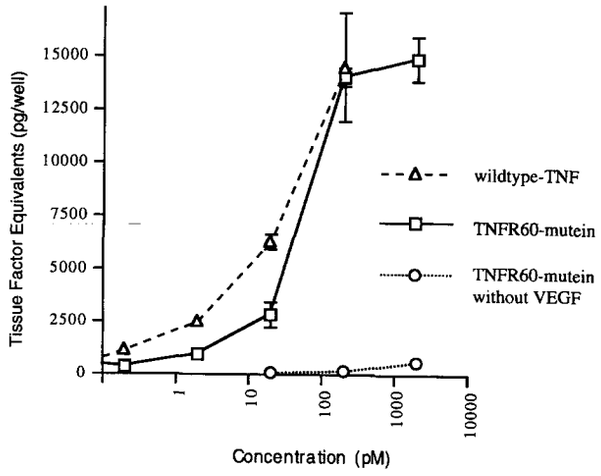

Fig. 1. Effect of a TNFR60-selective mutant on endothelial tissue factor production. Increasing concentrations of the TNFR60-selective receptor mutant TNFR32W-S86T (□) or wild-type TNF (△) were added, both in the presence of 2 nM VEGF. To demonstrate synergy, effects of the TNFR60-selective mutein in the absence of VEGF are also shown (○). Tissue factor was determined as described in the text. The mean of triplicates (± S.D.) are shown.

3. Results

3.1. Synergistic induction of tissue factor by VEGF and TNF receptor-selective TNF mutants

In the presence of VEGF already very low concentrations of TNF can induce tissue factor expression on the surface of human umbilical cord vein cells (HUVEC). We analyzed more closely the involvement of the two distinct TNF receptors in this system of TNF/VEGF synergy. Accordingly, all of the following experiments were performed in the presence of a constant optimal concentration of VEGF (2 nM), if not otherwise noted. In a first approach, we used mutants of the TNF molecule (muteins) which have previously been demonstrated to interact specifically with only one of the TNF receptors and hence represent receptor-selective tools [2,3,8]. The TNFR60-selective TNF mutant (TNFR32W-S86T) induced a dose-dependent production of tissue factor with a similar efficacy as wild-type TNF (Fig. 1). Of note, only at lower concentrations the induction of tissue factor by TNFR32W-S86T was less efficient when compared to the effects induced by wild-type TNF. Addition of the TNFR60-specific mutein in the absence of VEGF resulted in a much lower production of tissue factor, demonstrating that this mutein can efficiently synergize with VEGF (Fig. 1). In contrast, a TNFR80-specific mutein (TNFD143N-A145R) could not induce notable tissue factor production even at high concentrations up to 60 nM (Fig. 2). Bioactivity of the respective preparation of the TNF mutein had been controlled in a thymocyte proliferation assay [27], showing an only 5-fold lower bioactivity compared to wild-type TNF (data not shown). The highest TNFR80-specific mutein concentration used in this study exceeded the lowest active concentration tested in the thymocyte proliferation as-

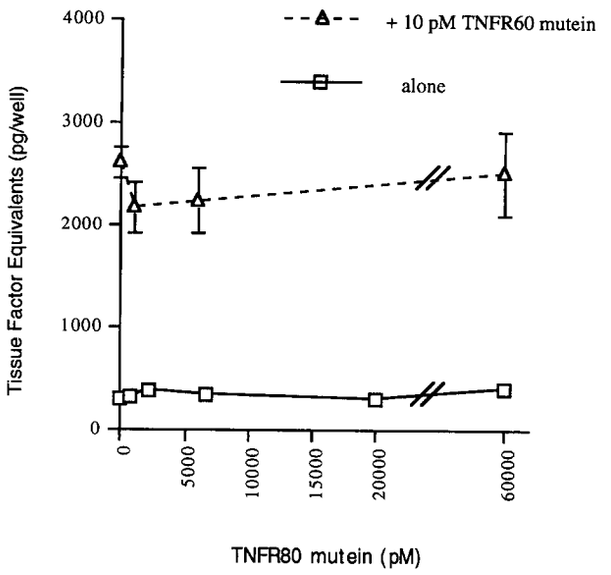

Fig. 2. Effect of a TNFR80-selective mutant on endothelial tissue factor production. Increasing concentrations of TNFR80-selective receptor mutant TNFD143N-A145R (□) were added together with 2 nM VEGF, alone or in the presence of a low dose (10 pM) of the TNFR60-selective receptor mutant TNFR32W-S86T (△). Tissue factor was measured as described in the text. The means of triplicates (±S.D.) are shown.

say about 100-fold. Furthermore, we tested the possibility that TNFR80-dependent signalling can be only observed in the presence of a costimulation of TNFR60. However, no enhancement of the cellular response was revealed by stimulation with the TNFR80-specific mutein in the presence of a suboptimal TNFR60 stimulation (Fig. 2). Similar results were obtained with a second TNFR80-specific mutein TNFD143F (data not shown).

3.2. *Analysis of TNF receptor expression*

The unresponsiveness of the HUVECs to the TNFR80-specific mutein was not due to lack of TNFR80 expression since cells of the same passage (passage two) expressed both TNF receptors as demonstrated by FACS analysis (Fig. 3) and [^{125}I]TNF binding composition studies (Table 1) using receptor-specific monoclonal antibodies. However, we observed that HUVECs tend to loose cell surface expression of TNFR80 upon prolonged cell culture (data not shown). Therefore, we used HUVECs of passage two throughout this study.

3.3. *Inhibition of VEGF–TNF synergy with neutralizing antibodies*

TNF receptor-specific neutralizing antibodies were investigated for their ability to influence the TNF-induced expression of tissue factor in the presence of VEGF. For this purpose HUVECs were preincubated with either TNFR60-specific monoclonal antibodies (H398) or with Fab fragments of TNFR80-specific purified polyclonal antibodies (M80). Blocking of TNF binding to TNFR60 completely abolished the TNF-mediated up-regulation of tissue factor at different TNF concentrations. This demonstrates that TNF binding to TNFR60 is essential for the synergy with VEGF at low (2 pM) as well as at high (40 and 400 pM) TNF concentrations (Fig. 4). In contrast, a crucial involvement of TNFR80 in this response seems to be limited to low TNF concentrations. Blocking of TNF binding to TNFR80 strongly inhibited the TNF effect at low TNF concentration (2 pM) and (although less prominent) at a TNF concentration of 40 pM. However, VEGF/TNF synergy at a high TNF concentration (400 pM) was hardly TNFR80 dependent (Fig. 4). Similar results were obtained with a neutralizing monoclonal anti-TNFR80 antibody (utr-1, data not shown). The decreased responsiveness of the TNFR80-specific antagonists at higher TNF concentrations was most probably not due to a lack in inhibition of TNF binding to TNFR80 because the same antibody efficiently blocked the TNF-induced, TNFR80-mediated proliferation of thymocytes [27] at TNF concentrations up to 1 nM (data not shown).

4. Discussion

In a previous study we have shown the principle capability of TNFR80 to induce tissue factor in HUVECs upon stimulation with membrane-bound TNF [27]. In the present study we focused on the interaction of the two TNF receptors with soluble TNF in the synergism of VEGF/TNF-induced tissue factor expression which has been implicated in TNF-induced tumor necrosis [18]. In particular, we were interested in the role of TNFR80 which was suggested to be involved in the process of tumor and scin necrosis [22,23]. The inability of TNFR80-selective TNF muteins to induce notable amounts of tissue factor in synergy with VEGF (in contrast to the effective TNFR60-specific mutant), demonstrates that TNFR80 is not able to synergize with VEGF per se upon engagement with soluble TNF. These results are unlikely to be due to an unsufficient receptor expression since we could demonstrate TNFR80-expression on the surface of HUVECs with almost the same levels as observed with the TNFR60. Moreover, a TNFR80-selective TNF mutein does not enhance TNFR60-induced responses. This argues against direct signalling of TNFR80, at least with respect to tissue factor induction.

As an additional possibility for cooperation of TNFR80 with TNFR60 we considered a ligand passing function of the TNFR80, first suggested by Tartaglia et al. [14]. In this model, the TNFR80, due to its higher on- and off-rates,

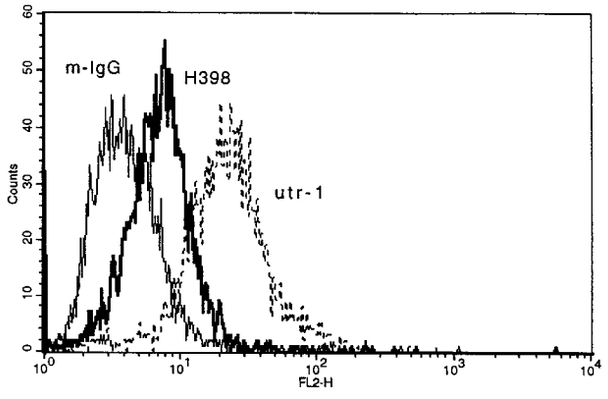

Fig. 3. Cytofluorometric determination of TNFR expression on HUVECs of passage two. Cells were incubated with mAb H398, specific for TNFR60 and with utr-1, specific for TNFR80. Further procedure and the measurements were performed as described in the text. Isotype-matched control antibodies (Co-IgG) were used to determine the background staining.

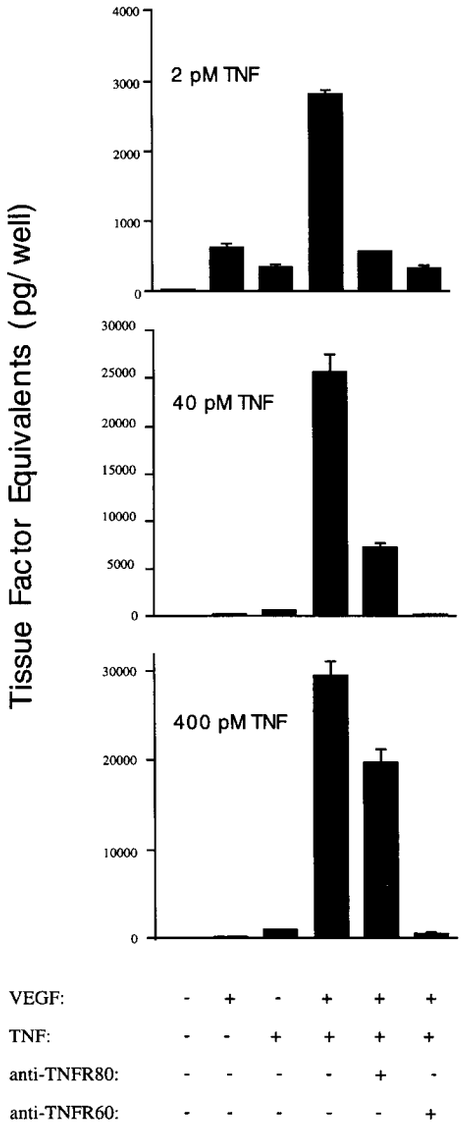

Fig. 4. Effect of neutralizing antibodies specific for either one of the TNF receptors. HUVECs were pretreated with either anti-TNFR80 (20 μg/ml) or anti-TNFR60 (10 μg/ml) antibodies. Different TNF concentrations as indicated were added together with 1 nM VEGF (TNF + VEGF). To demonstrate synergy, effects of VEGF (1 nM) or TNF (concentration as indicated) alone are also shown. Tissue factor was measured as described in the text. The means of triplicates (± S.D.) are shown.

passes the ligand to the signalling molecule TNFR60 possessing lower on- and off-rates. This mechanism should be important at low concentrations of TNF when TNFR60 signalling is limited by low ligand interaction due to the slow kinetics. In contrast, at higher concentrations of TNF, the supportive function of TNFR80 becomes less important because direct ligand binding to TNFR60 can take place. Indeed, our studies with antagonistic antibodies demonstrate that TNFR80 appears to be supportive for the TNF/VEGF synergism only at low concentrations of TNF, whereas TNF binding to TNFR60 is essential at all concentrations being tested. In a recent report, tissue factor production in HUVECs by TNF in the absence of VEGF was shown to be considerably less dependent on TNFR80 [5] as found here. These findings can be easily explained by the fact that in the presence of VEGF the minimum effective concentration of TNF is so dramatically lowered that TNFR60-mediated signalling is completely dependent on ligand passing by the TNFR80.

Because our findings suggest that TNFR80 enhances tissue factor production independent of intracellular signalling, it is most likely that some type of interaction between both receptors occurs. Besides the mechanism of ligand passing, it cannot be ruled out completely that the TNFR80 contributes to the observed synergy by forming receptor heteromers with the TNFR60. Studies using TNF receptor muteins can not address the question of heterodimerization, which should be observed with wild-type TNF only. However, in a previous study, using a different approach, heterodimerization of the two types of TNF receptor had not been observed [24]. In conclusion, this study suggests that low-dose TNF-mediated tissue factor induction on the endothelium of tumors producing significant amounts of VEGF is dependent on the TNFR80 and thus a possible candidate mechanism for the induction of necrosis [22,23]. The final evaluation of this aspect, however, is difficult because no data exist regarding the actual TNF concentration inside the tumor vasulature necessary to induce tumor necrosis.

The possible involvement in tumor necrosis induction may not be the only biological function of tissue factor production caused by the synergistic TNF/VEGF interaction. Expression of tissue factor in the endothelium of tumor vessels and within the tumor cells seems to correlate with the switch to the malignant and angiogenic phenotype of breast carcinomas [28]. In addition, tissue factor 'knockouts' in mice revealed an impaired pattern of extra-embryonic blood vessel formation during early embryogenesis (Peter Carmeliet, personal communication). In light of the fact that both TNF and VEGF can induce angiogenesis in vivo [29,30], a possible involvement of the here described synergistic tissue factor induction in angiogenic processes may be considered, especially when low concentrations of TNF and high concentrations of VEGF are present. Candidate pathological situations, when VEGF and its receptors are up-regulated, had been described for tumors [31,32], rheumatoid arthritis [33,34] and hypoxic lungs [35], but coexpression of TNF has not been studied in all of these examples. Further experimental in vivo data are required to test the hypothesis that TNF/VEGF-mediated tissue factor production is also involved in angiogenesis.

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