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Evidence that tumor necrosis factor-related apoptosis inducing ligand (TRAIL) inhibits angiogenesis by inducing vascular endothelial cell apoptosis

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ABSTRACT

Tumor necrosis factor (TNF) and its related ligands TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) play roles in the regulation of vascular responses, but their effect on the formation of new blood vessels (angiogenesis) is unclear. Therefore, we have examined the effects of these ligands on angiogenesis modeled with primary cultures of human umbilical vein endothelial cells (HUVEC). To examine angiogenesis in the context of the central nervous system, we have also modeled cerebral angiogenesis with the human brain endothelial cell line hCMEC/D3. Parameters studied were bromodeoxyuridine (BrdU) incorporation and cell number (MTT) assay (to assess endothelial proliferation), scratch assay (migration) and networks on Matrigel (tube formation). In our hands, neither TRAIL nor FasL (1, 10, and 100 ng/ml) had an effect on parameters of angiogenesis in the HUVEC model. In hCMEC/D3 cells by contrast, TRAIL inhibited all parameters (10–100 ng/ml, 24 h). This was due to apoptosis, since its action was blocked by the pan-caspase inhibitor zVADfmk (5×10^{-5} mol/l) and TRAIL increased caspase-3 activity 1 h after application. However FasL (100 ng/ml) increased BrdU uptake without other effects. We conclude that TRAIL has different effects on in vitro angiogenesis depending on which model is used, but that FasL is generally ineffective when applied in vitro. The data suggest that TRAIL primarily influences angiogenesis by the induction of vascular endothelial apoptosis, leading to vessel regression.

Introduction

Angiogenesis, or the formation of new blood vessels, is an essential component of many physiological and pathological processes including embryogenesis, wound healing, formation of blood supply to tumors or ischemic tissues, and as a driver of inflammatory processes. New vessels promote inflammation by increasing the sum total of inflammatory mediators produced by vascular endothelial cells and by increasing the surface area for leucocyte interactions. In the central nervous system (CNS), angiogenesis may play a role in diseases as diverse as glioma, multiple sclerosis and stroke [1–3].

Angiogenesis is studied by using in vitro models that examine the many processes that vascular endothelial cells participate in during the formation of new vasculature. These processes include endothelial proliferation, migration and tube formation. Also relevant is an understanding of how each of these processes are limited through induction of endothelial apoptosis, which leads to vessel regression. Inhibition of angiogenic vessels may prove a key strategy in limiting pathological angiogenesis, by cutting off blood supply to tumors, or by reducing harmful inflammation.

Vascular endothelial apoptosis in this context may be induced by death ligands. Death ligands are inducers of caspase dependent apoptosis through cognate binding to death receptors, and include members of the tumor necrosis factor (TNF) family of ligands including TNF itself, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL). TRAIL induces apoptosis by binding to its death receptors TRAIL-R1 and TRAIL-R2. TRAIL also binds to two decoy receptors TRAIL-R3 and TRAIL-R4, as well as the soluble receptor osteoprotegerin [4]. The binding of TRAIL to its death receptors results in activation of caspase-8. Through the extrinsic-death receptor mediated pathway, this causes direct cleavage of caspase-3 into active subunits that execute apoptosis [5]. Caspase-8 also activates the intrinsic-mitochondrial pathway, a more complex sequence of events involving mitochondrial membrane depolarization with release of cytochrome *c* which recruits Apaf1, ATP and caspase-9 to form an apoptosome. Here the cleavage product of caspase-9 results in cleavage and activation of caspase-3 to execute apoptosis [6].

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In this study, we have modeled angiogenesis using two in vitro models. Most work on in vitro angiogenesis has been conducted in human umbilical vein endothelial cells, but previous reports on the action of TRAIL are contradictory [7,8]. Therefore we examined the effects of both TRAIL and FasL in this model. There are to our knowledge, no previous reports on the effect of these ligands in models of cerebral angiogenesis. So, cerebral angiogenesis was modeled with hCMEC/D3 cells, a recently described human brain endothelial cell line [9].

Material and methods

Cell cultures. The human brain endothelial cell line hCMEC/D3 was kindly provided by Dr. B. Weksler (Weill Medical College, NY). The cells were recovered from liquid nitrogen at the 4th passage after subcultivation, and plated onto rat tail collagen (type I, BD Biosciences) in complete EGM-2MV medium (Microvascular Endothelial Cell Medium-2, Lonza/Cambrex). This consisted of EGM-2MV medium with added 5% fetal bovine serum (FBS) and a supplement containing hydrocortisone, ascorbate, gentamycin and the growth factors VEGF, IGF-1, EGF, and bFGF. The medium was replaced every 2–3 days, with cells reaching confluency after 7–10 days. Cells were passaged using trypsin–EDTA (1×) for subsequent use in experiments. Primary cultures of human umbilical vein endothelial cells (HUVEC) from a single donor were purchased from Lonza/Cambrex and used at passage 3 or 4. Cells were cultured on plastic coated with rat tail collagen in EGM-2 medium containing 5% FBS (Lonza/Cambrex).

Materials. Reagents were obtained from the following sources: non tagged soluble recombinant human TRAIL (amino acids 114–281) was from Peprotech, Fas ligand (FasL) and enhancer from Alexis Biochemicals, TNF from Sigma, and the pan-caspase inhibitor zVADfmk from R&D. During experiments, FasL was combined with enhancer (1 μg/ml). Vascular endothelial growth factor (VEGF) was from R&D and basic fibroblast growth factor (bFGF) was obtained from Sigma.

Flow cytometry. Flow cytometric analysis was performed by FACSCalibur (Becton–Dickinson), as previously described [10,11]. Flow cytometric antibodies were IgG_1 phycoerythrin-conjugated monoclonal mouse anti-human antibodies against TRAIL-R1, R2, R3 and R4, TRAIL, FasL (eBiosciences), Fas (BD Biosciences), VEGF-R1 and R2 (R&D) and IgG_1 isotype control (eBioscience).

Proliferation and cell number assays. Proliferation was assessed from incorporation of bromodeoxyuridine (BrdU) using a colorimetric BrdU cell proliferation ELISA kit (Roche Applied Science). Briefly, 5×10^3 cells were added to each well of a 96-well plate and left to attach in complete medium for 24 h. In hCMEC/D3, cells were then placed in basal medium without serum but containing 2% bovine serum albumin (BSA) overnight (14–18 h) which renders the cells quiescent. Ligands were then applied in this medium for 24 h with BrdU labeling in the final 4 h (10^{-5} mol/l) at 37 °C. In HUVEC, experiments were carried out in ordinary culture medium containing 5% FBS. After ligand application, in both cell types, medium was removed and replaced with a fixation and denaturing solution followed by peroxidase conjugated Fab fragments against BrdU, reacted with tetramethyl-benzidine substrate to develop color, measured as absorbance at 450 nm with a plate reader (BioRad). Cell numbers were assessed with the colorimetric MTT assay (Cell proliferation kit I, Roche Applied Science). This assay relates cell viability to the metabolic reduction of the tetrazolium salt MTT to a purple reaction product. Briefly, cells were seeded and manipulated as described for the BrdU assay. At the end of ligand treatments (24 h), the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each well for 4 h, a solubilizing solution was added overnight, and absorbance at 570 nm was measured with a plate reader.

Cell migration scratch assay. The scratch assay was carried out as described [12]. 2.5×10^5 cells were plated on wells of a 6-well plate and grown to confluency (2–3 days). A 200 μl plastic pipette tip was drawn across the wells to create two linear regions denuded of cells. Wells were washed and ligands added in 2% BSA (hCMEC/D3) or 5% FBS (HUVEC). Initial images of the denuded zone were collected using an inverted microscope (Leica Canada, DM IL) attached to a digital camera (Canon A630). Plates were then incubated at 37 °C for 24 h (hCMEC/D3) or 18 h (HUVEC). Post-treatment images of the same fields were captured and analyzed using imaging software (NIH Image J, version 1.41o) to count cells that migrated into the scratched region.

Tube formation assay. Individual wells of a 48-well plate were coated with100 μl of Matrigel (growth factor depleted; BD Biosciences). Ligands were then added to the wells at 37 °C for 40 min in order to saturate the Matrigel before adding 4×10^4 cells in serum free medium containing 2% BSA (for both HUVEC and hCMEC/D3). Cells and ligands were then incubated together for varying periods as indicated in the Results. At each time point, 5 fields were photographed with a digital camera attached to an inverted microscope; these were located in the center of each well and each of four quadrants (250× magnification). The images were analyzed using imaging software (NIH Image J).

Caspase-3 activity assay. Caspase-3 activity was detected with a fluorometric immunosorbent enzyme assay kit (caspase-3 activity assay, Roche Applied Science). Briefly, hCMEC/D3 were grown to confluency in 6-well plates and treated with ligands for 1, 3, or 6 h in serum free medium containing 2% BSA. 2×10^6 cells per treatment were lysed and cell supernatants (100 μl) added to wells coated with a monoclonal antibody to caspase-3. A reagent was then added that undergoes proteolytic cleavage in proportion to the amount of activated caspase-3 to generate a fluorescent product read as absorbance at 505 nm with a plate reader.

Statistical analysis. Differences between mean values were analyzed by one way analysis of variance followed by the Bonferroni test, using statistical software (GraphPad Prism). $P < 0.05$ was taken as significant.

Results

Effects of TRAIL, Fas ligand and TNF in HUVEC

The result of the BrdU incorporation, cell number and migration assays, is shown in Fig. 1. As a positive control, 24 h treatment with vascular endothelial growth factor (VEGF, 10 ng/ml) and basic fibroblast growth factor (bFGF, 10, 50 ng/ml) induced a significant increase in BrdU incorporation (Fig. 1A). bFGF at these doses also induced a significant increase in cell numbers (Fig. 1B). VEGF and bFGF (both 100 ng/ml) also significantly increased cell migration over 24 h (Fig. 1C). TRAIL, FasL (both 1, 10, and 100 ng/ml) and TNF (10, 100 U/ml) had no effect on these parameters (Fig. 1A–C). Tube formation was also analyzed in HUVEC (Fig. 2). Representative images are shown in Fig. 2A. The data are expressed as the average length or average area of the tubular structures. Under control conditions, average area increased slightly over 24 h, while average length remained stable. VEGF (50 ng/ml) induced significant increases in both parameters over 24 h (Fig. 2B and C). FasL (1, 10, and 100 ng/ml), TRAIL (1, 10, and 100 ng/ml) and TNF (1, 10, and 100 U/ml) had no effect (the effect of individual doses is shown in Fig. 2B and C). These data show that TRAIL, FasL, and TNF have no effect on parameters of angiogenesis modeled with HUVEC.

Effect of TRAIL, Fas ligand, and TNF in hCMEC/D3

Flow cytometry was carried out to detect receptors for TRAIL and FasL on hCMEC/D3 cells. The fluorescence intensity of labeling

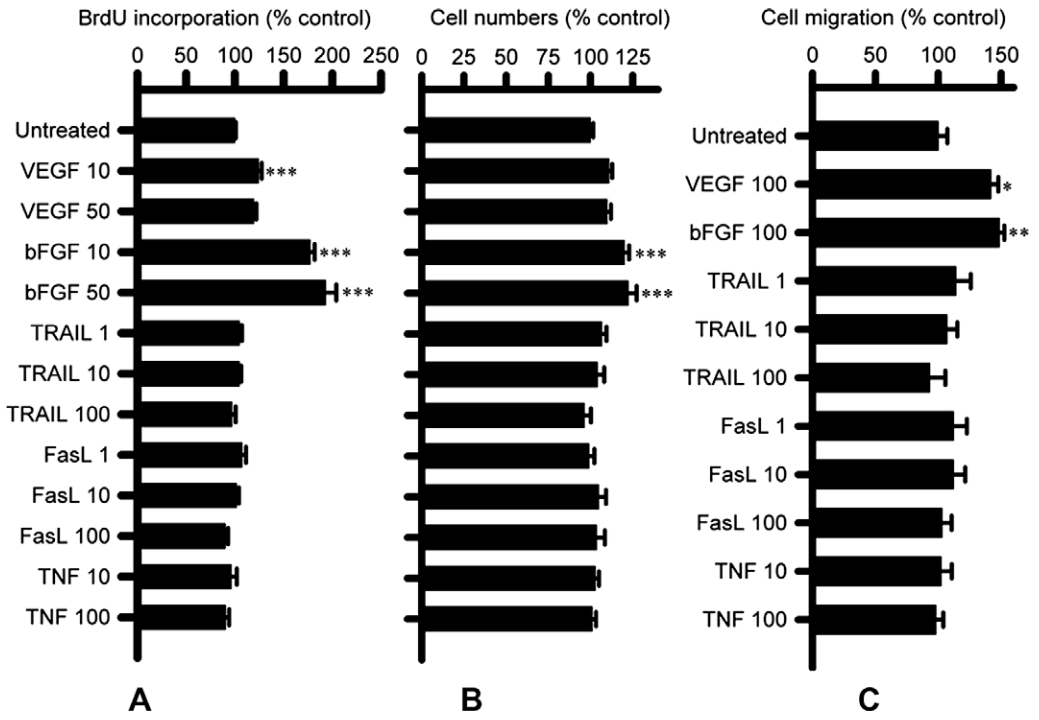

Fig. 1. Effects of multiple factors on parameters of angiogenesis in the HUVEC. Data are shown for BrdU incorporation (A), cell number analysis by MTT assay (B) and cell migration by scratch assay (C). Cells were treated (24 h) with vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), TNF-related apoptosis inducing ligand (TRAIL), Fas ligand (FasL), and tumor necrosis factor (TNF). Numbers indicate doses in ng/ml except for TNF where dosage is U/ml. Data are normalized to the untreated control, and shown as mean ± standard error of the mean (SEM). Significance is compared to the untreated control; only significant differences are marked ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). In (A,B), $n = 24$ for untreated cells and $n = 12$ for other groups. In (C), $n = 12$ for untreated cells, $n = 6$ for other groups.

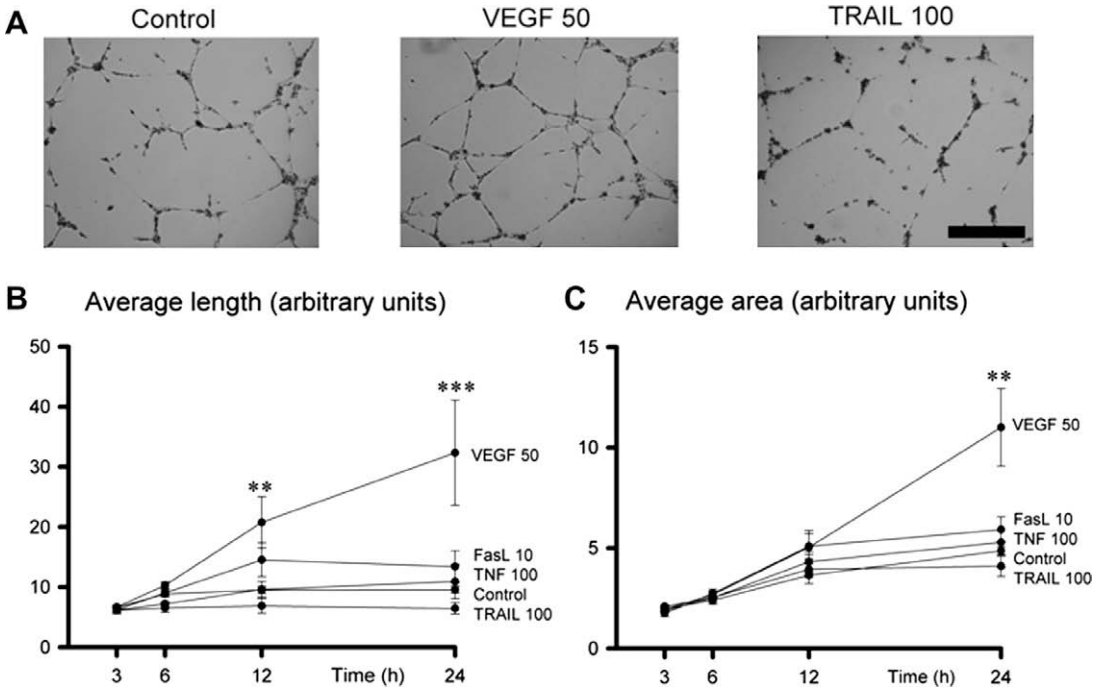

Fig. 2. Tube formation assay on Matrigel in HUVEC treated with growth factors and indicated ligands. Doses are ng/ml except TNF (U/ml). Representative images are shown (A) taken at 20x magnification (scale bar = 500 μm). Data (mean ± SEM) are expressed as average length (B) or average area (C) of tubular structures over time. Relative changes are shown (expressed in arbitrary units). Significance is compared to the untreated control and only significantly different results are shown ($^{**}P < 0.01$, $^{***}P < 0.001$). In (B and C), $n = 8$ for controls and $n = 4$ for all other groups.

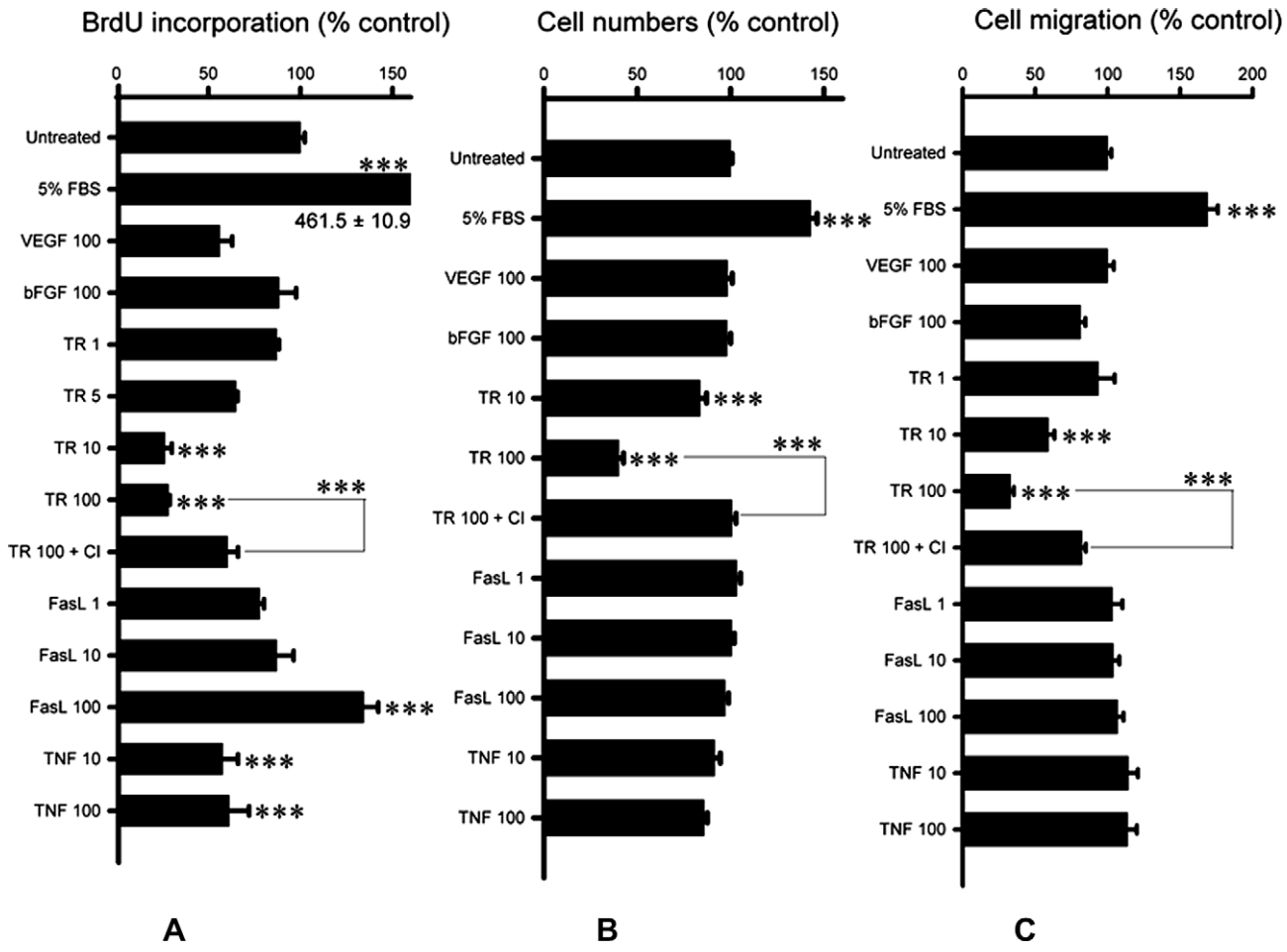

Fig. 3. Effects on parameters of cerebral angiogenesis modeled with hCMEC/D3. Data are shown for BrdU incorporation (A), cell number analysis (B), and cell migration (C). Cells were treated (24 h) with growth factors and indicated ligands. In some experiments, TRAIL was combined with the caspase inhibitor zVADfmk (5×10^{-5} mol/l, CI). Numbers indicate doses in ng/ml except for TNF (U/ml). Data are normalized to the untreated control, and shown as mean ± SEM. Significance is compared to the untreated control except where indicated by cross bars; only significant differences are marked ($^{***}P < 0.001$). In (A), $n = 32$ for untreated cells, $n = 8$–12 for 5% FBS and growth factors and $n = 4$–20 for other ligands. In (B), $n = 60$ for untreated cells, $n = 26$ for 5% FBS, $n = 24$ for growth factors, and $n = 12$–36 for other ligands. In (C), $n = 79$ for untreated cells, $n = 63$ for 5% FBS, and $n = 15$–40 for other ligands.

with specific antibodies was plotted as a function of cell number (data not shown). The isotype control antibody staining was gated to give a value of about 10% of the cells (9.93 ± 0.17%, mean ± SEM, $n = 9$). This allows the relative increase in staining for other antibodies to be expressed as % of cells within the set range. Compared to ca. 10% staining in control cells, about 90% of cells stained for TRAIL-R2 (90.76 ± 2.00%, $n = 5$, $P < 0.001$ compared to isotype control). This was considerably greater than staining for TRAIL-R3 (17.20 ± 0.31%, $n = 5$, $P < 0.001$ compared to control), with no significant increase above control for either TRAIL-R1 (12.12 ± 0.25%, $n = 5$) or TRAIL-R4 (9.75 ± 1.00%, $n = 4$). The Fas receptor was also detected in a high percentage of cells (63.98 ± 1.79%, $n = 5$, $P < 0.001$), but there was no detectable Fas ligand (FasL) or TRAIL (means under 3%, not significantly increased). There was also expression of the VEGF receptors VEGF-R1 (20.1 ± 0.36%, $n = 4$, $P < 0.001$) and VEGF-R2 (66.18 ± 0.36%, $n = 4$, $P < 0.001$). The hCMEC/D3 line has an ordinary requirement for medium containing multiple growth factors. We therefore anticipated that single growth factors would be insufficient to act as positive controls. Indeed, VEGF and bFGF (10, 50, and 100 ng/ml) applied singly or in combination with the co-factor heparin (50 mg/ml plus 100 U/ml heparin, data not shown) had no effect on BrdU incorporation, cell numbers or migration (effect of 100 ng/ml shown in Fig. 3). However, introduction of 5% FBS to cells rendered quiescent (in serum free medium containing 2% BSA) stimulated a strong positive response, that served as a positive control (Fig. 2A–C). Quiescent cells responded differently to TRAIL and FasL. TRAIL (1–100 ng/ml) induced a dose dependent reduction in BrdU incorporation (Fig. 3A), that was inhibited by co-treatment (100 ng/ml TRAIL) with the pan-caspase inhibitor zVADfmk (5×10^{-5} mol/l). A similar reduction was observed in cell numbers and migration, also blocked by zVADfmk co-treatment (Fig. 3B and C). In contrast, FasL (1, 10 ng/ml) had no effect on BrdU uptake, while 100 ng/ml had a small stimulatory effect (Fig. 3A). TNF (10, 100 U/ml) mimicked the inhibitory effect of TRAIL on BrdU incorporation (Fig. 3A). Both FasL and TNF had no effect on cell numbers or migration (Fig. 2B). To confirm the impression that TRAIL induced apoptosis in the cells, caspase-3 activity was measured. TRAIL induced a significant increase in caspase-3 activity over untreated cells at 1 h (2.10 ± 0.35 vs control 0.10 ± 0.01, arbitrary fluorescence units, $n = 4$, $P < 0.001$ compared to control), with values returning to baseline by 3 h (0.73 ± 0.23, $n = 4$) and 6 h (0.50 ± 0.22, $n = 4$).

Tube formation on Matrigel was assessed for periods between 3 and 24 h. Representative images are shown in Fig. 4A. These images show that the tubular structures increased in both length and area but reduced in number as the assay progressed. The individual regions were therefore analyzed in terms of average length and average area (Fig. 4B and C). Untreated cells (control data)

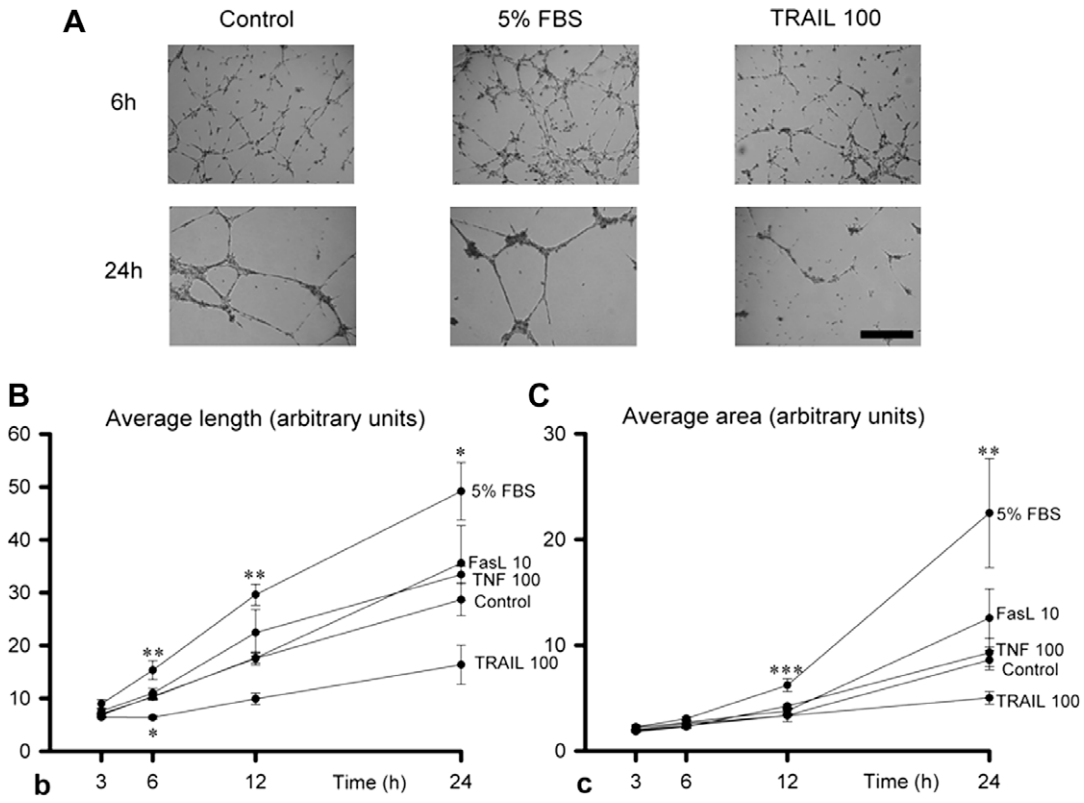

Fig. 4. Tube formation assay on Matrigel in hCMEC/D3 treated with serum (5% FBS) and indicated ligands. Doses are ng/ml except for TNF (U/ml). Representative images are shown (A) taken at 20x magnification (scale bar = 500 μm). Data (mean ± SEM) are expressed as average length (B) or average area (C) of tubular structures over time. Relative changes are shown (expressed in arbitrary units). Significance is compared to the untreated control and only significantly different results are shown ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). In (B,C), $n = 8$ for controls and $n = 4$ for all other groups.

showed a progressive increase in both parameters over time. This was accelerated by the introduction of 5% FBS as a positive control (Fig. 4B and C). 50 ng/ml doses of VEGF and bFGF had no significant effect on either parameter (data not shown) while TRAIL (100 ng/ml) reduced both parameters, inducing a statistically significant reduction in average length at 6 h (Fig. 4B). FasL (10 ng/ml) and TNF (100 U/ml) had no effect (Fig. 4B and C). The data confirm that tube formation in hCMEC/D3 cells is inhibited by TRAIL, and unaffected by other ligands.

Discussion

Effect of TRAIL in HUVEC

TRAIL was initially reported to promote apoptosis in cancer cells without harming normal cells, and it remains a promising candidate for novel anti-cancer therapy [13]. Physiologically, its best characterized function is in tumor surveillance [14]. In the vasculature, TRAIL is expressed by vascular smooth muscle [15] and may function in a paracrine function to regulate vascular endothelial functioning by inducing endothelial production of nitric oxide and prostanoids [16]. In an inflammatory context, TRAIL is upregulated by virtually all immune cells including neutrophils, T and B lymphocytes, natural killer cells, dendritic cells and monocytes [17]. There are different outcomes in previous studies of TRAIL and angiogenesis in peripheral endothelial cells. In HUVEC, TRAIL was reported to induce activation of Akt and ERK leading to endothelial proliferation [18] and to increase cell migration and tube formation without altering endogenous endothelial expression of VEGF [7]. TRAIL had no effect on angiogenesis in the rat aortic ring model [19]. TRAIL was reported to inhibit cancer related angiogenesis in an implant of PC-3 tumor cells in mice, with a reduction in local VEGF expression [20]. Finally, in contrast to the previous finding that TRAIL is stimulatory [7], TRAIL was found to inhibit tube formation, cell numbers and endogenous VEGF expression in HUVEC in another study [8]. The pro-angiogenic action of TRAIL [7] was induced by 10 ng/ml TRAIL linked to a histidine tag, while the anti-angiogenic effect of TRAIL [8] was induced by higher doses (100–400 ng/ml). Tagged forms of TRAIL may behave differently from untagged forms, as for instance when histidine tagged TRAIL induced apoptosis in hepatocytes that was not seen with the untagged moiety [21,22]. The data in this study lead us to conclude that both TRAIL and FasL have no effect on parameters of angiogenesis modeled in HUVEC (Figs. 1 and 2). We speculate that this could relate to our use of untagged TRAIL instead of histidine tagged TRAIL [7], since other conditions (doses of TRAIL—10 ng/ml in [7], 100 ng/ml in [8], presence of serum in both studies) have been replicated in this work.

Actions of TRAIL and FasL in hCMEC/D3

This is to our knowledge, the first study to examine the actions of TRAIL and FasL in a model of cerebral angiogenesis. In hCMEC/D3 cells, TRAIL induced a dose dependent reduction in BrdU uptake, cell numbers, migration and tube formation (Figs. 3 and 4). The effect of TRAIL was inhibited in combination with the pan-caspase inhibitor zVADfmk (Fig. 3). To confirm that TRAIL inhibited these processes through induction of apoptosis, TRAIL was found to induce an early (1 h) increase in caspase-3 activity. TNF also reduced BrdU uptake (Fig. 3) but had no effect on migration (Fig. 3) or tube formation (Fig. 4). In contrast, Fas ligand had minimal effects, inducing a small but significant increase in BrdU uptake (Fig. 3), without effect on other parameters (Figs. 3 and 4). The propensity of hCMEC/D3 cells to undergo apoptosis in response to

TRAIL is not the result of serum withdrawal, since TRAIL was also effective in reducing BrdU uptake, cell numbers and migration when 5% FBS was present (data not shown). Also, FasL did not induce an apoptotic response in the cells, so there is no general propensity for hCMEC/D3 cells to undergo apoptosis in response to death ligands. However, a study in primary cultures of human brain endothelial cells found that TRAIL does not induce apoptosis [23]. This suggests that the human brain endothelial cells used in this study have been sensitized to TRAIL by the process of immortalization. This is not irrelevant to an understanding of the role that TRAIL may play in regulating cerebral angiogenesis. Other evidence indicates that TRAIL only induces apoptosis in vascular endothelial cells when they have been previously sensitized. This may be relevant to understanding the balance between pro- and anti-angiogenic factors in limiting angiogenesis. Thus, TRAIL induces apoptosis in HUVEC only when there is inhibition of phosphatidylinositol 3-kinase and its downstream activation of the serine/threonine kinase, Akt [10,18]. TRAIL (but not FasL) also induces apoptosis in unmodified EA.hy926 cells, an immortalized cell line derived from HUVEC. In this context the endothelial cells become pro-adhesive for neutrophils [11]. TRAIL promotes apoptosis of human dermal microvascular endothelial cells when they are grown on a substrate that inhibits integrin signaling [24]. Angiogenesis induced by VEGF acts through Akt activation and can be blocked when Akt is inhibited by various anti-angiogenic factors such as angiotensin II and thromboxane A2 [25,26]. Thus, factors that inhibit angiogenesis could sensitize vascular endothelium to undergo apoptosis by TRAIL, leading to vessel regression. However, TRAIL would not be expected to induce apoptosis in the normal microvasculature.

Conclusions

The data in this study suggest that TRAIL (with supportive evidence for TNF) may inhibit cerebral angiogenesis by inducing apoptosis in appropriately sensitized cerebral endothelial cells, and so exert a potential anti-inflammatory role in the human CNS. Fas ligand may be neutral, or even promote cerebral angiogenesis. The combination of our findings in HUVEC and hCMEC/D3 suggests that TRAIL principally influences angiogenesis by inducing apoptosis in vascular endothelial cells, leading to vessel regression. In contrast to other in vitro studies [7,18], we did not find evidence of a pro-angiogenic role for TRAIL.

Acknowledgments

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