Tumor Necrosis Factor-α Promotes Macrophage-Induced Vascular Smooth Muscle Cell Apoptosis by Direct and Autocrine Mechanisms

Joseph J. Boyle, Peter L. Weissberg, Martin R. Bennett

Objective—We have previously shown that human macrophages induce human plaque vascular smooth muscle cell (VSMC) apoptosis by cell-cell proximity, Fas-L, and nitric oxide (NO), thereby predisposing to plaque rupture. This study sought to analyze whether tumor necrosis factor-α (TNF-α) contributes additionally to macrophage-induced VSMC apoptosis.

Methods and Results—Macrophage-induced VSMC apoptosis was examined in direct coculture. Antagonistic antibodies to TNF-receptor (R1) inhibited VSMC apoptosis, and preincubation of monocytes and VSMCs indicated that TNF-R1 on both cell types contributed to macrophage-induced VSMC apoptosis. Correspondingly, both monocytes and VSMCs expressed TNF-R1, and macrophages expressed cell surface TNF-α. Two NO donors upregulated VSMC surface TNF-R1, and exogenous TNF-α induced VSMC apoptosis synergistically with the NO donor diethyleneetriamine/NO, indicating that NO sensitizes VSMCs to TNF-α. Neutralizing anti–TNF-R1 antibodies inhibited macrophage activation assessed by Fas-L expression and NO secretion.

Conclusions—TNF-α promotes macrophage-induced VSMC apoptosis by autocrine and direct pathways. (Arterioscler Thromb Vasc Biol. 2003;23:1553-1558.)

Key Words: macrophages ■ plaque rupture ■ vascular smooth muscle cells ■ apoptosis ■ tumor necrosis factor

Atherosclerotic plaque rupture causes myocardial infarction.1-3 Plaque ruptures are associated with increased fibrous cap macrophages, reduced fibrous cap vascular smooth muscle cells (VSMCs), and increased VSMC apoptosis.3-5 Because VSMCs are the only cells within plaques capable of synthesizing structurally important collagen isoforms, VSMC apoptosis might promote plaque rupture.4,5

We have shown previously that cultured, human blood monocyte-derived macrophages induce human VSMC apoptosis by direct cell-cell contact, Fas-L/Fas, and nitric oxide (NO).6,7 Macrophages produce other proapoptotic factors, including the Fas-L homologue tumor necrosis-factor-α (TNF-α).5 TNF-α, like Fas-L, has a bioactive, membrane-bound pro-form that mediates cell-cell contact–dependent apoptosis both in vitro6,9 and in vivo.10 Although the mechanism by which TNF-α acts in cytotoxicity is less clear, there is evidence that it induces VSMC apoptosis synergistically, with the inflammatory cytokines interleukin-1β and interferon-γ.11

TNF-α acts through 2 receptors, TNF-R1 and TNF-R2.12 Both TNF-R1 and TNF-R2 are homologous to Fas.13,14 Like Fas, TNF-R1 has a death domain that initiates assembly of a death-induced signaling complex, thus activating caspases.13 TNF-R2 is homologous to TNF-R1 and Fas but lacks a death domain.13 The mechanism for the proapoptotic effect of TNF-R2 is uncertain. TNF-R2 might, like TNF-R1, by way of tumor necrosis factor receptor-associated factor (TRAF) adaptor molecules,13 produce proapoptotic effects. Indeed, TNF-R1 and TNF-R2 cooperatively interact to induce apoptosis through TRAFs.15 Alternatively, Grell et al.12 have postulated that TNF-R2 might mediate the proapoptotic effects of TNF by ligand passing to TNF-R1. Furthermore, macrophage-derived TNF-α might activate macrophages in an autocrine loop.16-18 Although this could promote cytotoxicity, it is uncertain whether TNF-R1 or TNF-R2 mediates this process.

We tested the hypothesis that TNF-α contributes to macrophage-induced VSMC apoptosis. We show herein that macrophage-derived TNF-α contributes to macrophage-induced apoptosis through effects on VSMCs and autocrine macrophage activation. Importantly, NO could directly sensitize VSMCs to TNF-α by increasing cell surface TNF-R1.

Methods

Cell Culture and Analysis of Apoptosis and Protein Expression

Human peripheral blood macrophages and human carotid, coronary medial, and aortic VSMCs were isolated and cultured as before (please see http://atvb.ahajournals.org).5

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Antagonists of TNF-R1 and TNF-R2 Inhibit Macrophage-Induced VSMC Apoptosis

To test whether TNF-R1 or TNF-R2 was involved in macrophage-induced VSMC apoptosis, macrophages and VSMCs were cocultured for 8 days, and VSMC apoptosis was then assessed by using DNA-binding dyes and the macrophage marker CD14 (conjugated to fluorescein isothiocyanate [FITC]). This allows discrimination of apoptosis in both macrophages and VSMCs. We assessed both human carotid plaque–derived VSMCs and a semi-immortalized, human medial VSMC cell line, HCMED1-E6 VSMCs.6

Human plaque VSMCs showed low levels of spontaneous apoptosis (5.2±0.9%), but macrophage coculture significantly increased the plaque VSMC apoptotic index (81±2.9%) (Figure 1A). Neutralizing antibodies to TNF-R1 significantly inhibited macrophage-induced VSMC apoptosis (32±4.2%), with no effect observed with an isotype-matched control (83±2.8%). Anti–TNF-R1 produced no change in apoptosis of isolated macrophages or VSMCs individually (P>0.05, data not shown).

HCMED1-E6 VSMCs showed a similar pattern of macrophage-induced VSMC apoptosis (Figure 1B; control VSMCs, 7.6%±2.9%; untreated macrophage/VSMC cocultures, 76%±5.5%; anti–TNF-R1 antibody, 4.9%±0.9%; anti–TNF-R2 antibody, 2.5%±0.4%; and isotype control antibody, 82±9.8%) Thus, anti–TNF-R1 and anti–TNF-R2 antibodies reduced macrophage-induced HCMED1-E6 VSMC apoptosis, implying that both pathways are required for full macrophage cytotoxicity.

Expression of TNF-α/TNF-R1/TNF-R2 Signaling Pathway in Monocytes, Macrophages, and VSMCs

Because macrophages and VSMCs were directly cocultured, inhibition of the macrophage-induced VSMC apoptosis by TNF-R antagonists could be mediated by effects on macrophages, VSMCs, or both. To clarify which cells were the site of action, we assessed expression of TNF-α, TNF-R1, and TNF-R2 in monocytes (culture day 1), macrophages (culture day 8), B. Flow cytometric detection of TNF-α, TNF-R1, and TNF-R2 for surface expression (unpermeabilized cells) or total expression (permeabilized cells). histograms are shown for macrophages (culture day 1) or macrophages (culture day 8). Shaded histograms represent staining with isotype control (background); open histograms represent specific staining. C, Time course of expression of surface TNF-α on macrophages. D, Flow cytometric detection of TNF-α, TNF-R1, and TNF-R2 for surface expression (unpermeabilized cells) or total expression (permeabilized VSMCs). HCMED1-E6 VSMCs, aortic VSMCs (passage 4, n=4 donors), and plaque VSMCs (passage 1, n=3 donors) were tested.
Macrophage Maturation Increases Surface TNF-α
We have previously shown that VSMC apoptosis induced by direct coculture with macrophages at day 8 after isolation requires direct cell-cell contact or proximity.6 We reasoned that to effect cell-cell contact–related apoptosis, TNF-α and TNF-R1 must be expressed on the cell surface. Although more difficult to demonstrate, detection of TNF-α on the cell surface would be more informative for contact-dependent apoptosis than detection by ELISA or Western blots of surface TNF-R1.

Because TNF-R1 was found on cultured macrophages and VSMCs, we asked whether TNF-R1 antibodies reduced VSMC apoptosis. We have recently found that macrophage-induced VSMC apoptosis involves NO-induced cell surface trafficking of Fas.19 We therefore asked whether TNF-R1 underwent a similar relocalization. To examine total and surface expression of TNF-R1, we performed flow cytometry of permeabilized and nonpermeabilized VSMCs, respectively (Figure 2D). TNF-R1 was detected in permeabilized but not in nonpermeabilized HCMED1-E6 VSMCs, indicating an intracellular location. TNF-R2 could not be detected on either permeabilized or nonpermeabilized HCMED1-E6 VSMCs, consistent with data from the Western blots.

In 4 of 5 isolates of (passage 4) normal human aortic medial VSMCs, TNF-R1 was detected only after permeabilization, indicating that it was located mainly intracellularly (Figure 2D). Thus, aortic VSMCs expressed TNF-R1 mainly intracellularly, similar to HCMED1-E6 VSMCs.

In contrast, TNF-R1 was detected in nonpermeabilized, carotid plaque VSMCs (n=3 donors), indicating that it was on the cell surface (Figure 2D). No additional TNF-R1 expression was detected after permeabilization (Figure 2D), indicating that in contrast to HCMED1-E6 VSMCs and aortic VSMCs, TNF-R1 is found mainly on the surface of carotid plaque VSMCs. The VSMCs examined did not express TNF-α (Figure 2D).

Aortic and HCMED1-E6 VSMCs Express TNF-R1 Intracellularly but Plaque VSMCs Express Cell Surface TNF-R1
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Signaling Through Both Macrophages and VSMC TNF-R1 Contributes to Macrophage-Induced VSMC Apoptosis
Because TNF-R1 was found on cultured macrophages and VSMCs, we asked whether TNF-R1 antibodies reduced apoptosis by acting on macrophages or VSMCs. Plaque VSMCs and macrophages were selectively preincubated with anti–TNF-R1 before coculture, and apoptosis was assessed as before (Figure 3A and 3B). Preincubation of either macrophages or plaque VSMCs with anti–TNF-R1 inhibited macrophage-induced apoptosis (Figure 3). This indicates that both macrophage TNF-R1 and plaque VSMC TNF-R1 contributed to macrophage-induced plaque VSMC apoptosis.

The contribution of HCMED1-E6 VSMC-TNF-R1 to macrophage-induced VSMC apoptosis was assessed. The protocol was modified to account for the intracellular HCMED1-E6 VSMC TNF-R1’s being difficult to neutralize with preincubated antibody and macrophage TNF-R1’s being undetectable at culture days 6 to 8 (Figure 3B). We compared adding anti–TNF-R1 antibodies to macrophages at culture days 1 to 6 with adding anti–TNF-R1 antibodies to coculture at culture days 6 to 8 (Figure 3B). Anti–TNF-R1 reduced VSMC apoptosis to 10% to 20% of control value when added either to macrophages (culture days 1 to 6) or to cocultures (Figure 3B). Thus, macrophage TNF-R1 and VSMC TNF-R1 contributed to macrophage-induced apoptosis of both plaque VSMCs and coronary artery–derived VSMCs. Because anti–TNF-R1 antibodies were effective even when preincubated with macrophage monocultures and then washed off, we concluded that TNF-α activated macrophages to a proapoptotic phenotype by way of TNF-R1.

NO Donors Upregulate VSMC Surface TNF-R1
The NO donors diethylenetriamine (DETA)/NO and sodium nitroprusside upregulate VSMC surface Fas death receptor from an intracellular pool, contributing to macrophage-apoptosis.
induced apoptosis.\textsuperscript{19} We therefore tested whether the same applied to VSMC TNF-R1. DETA/NO (1 mmol/L) and sodium nitroprusside (1 mmol/L) upregulated surface TNF-R1 on HCMED1-E6 VSMCs and on primary, untransfected, aortic medial VSMCs, as detected by 2 anti–TNF-R1 antibodies (online Figure IA and IB; please see http://atvb.ahajournals.org). The control compound DETA produced no change in VSMC surface TNF-R1, indicating specificity for NO (online Figure I).

**TNF-α Induces Apoptosis in Cultured, Plaque-Derived VSMCs**

If TNF-α–induced VSMC apoptosis occurs by a direct effect on VSMCs, then exogenous TNF-α should induce VSMC apoptosis. Furthermore, if NO-induced upregulation of TNF-R1 is functionally effective, then NO treatment should sensitize VSMCs to TNF-α–induced apoptosis. This was tested on plaque-derived VSMCs (online Figure IC). Both DETA/NO and TNF-α (100 nmol/L) increased plaque VSMC apoptosis, with a significant further increase when both agents were used (61±2.3%; Figure IC). Thus, TNF-α can induce apoptosis of human plaque VSMCs on its own. Furthermore, NO sensitizes plaque VSMCs to TNF-α–induced apoptosis.

We next tested whether TNF-α and Fas-L were synergistic for inducing plaque VSMC apoptosis, as assessed by propidium iodide/Hoechst staining after 48-hour incubation (online Figure ID). With a lower concentration of TNF-α (10 nmol/L), neither TNF-α nor Fas-L induced apoptosis alone, but the combination did. This results upheld the hypothesis that Fas-L and TNF-α induce plaque VSMC apoptosis synergistically.

**Signaling Through TNF-R1 and TNF-R2 Is Required for Macrophage Activation to Express Fas-L and NO**

In culture, human blood–derived macrophages express Fas-L and NO and become proapoptotic in parallel with the upregulation of the recognized activation markers CD16 and human leukocyte antigen-DR (HLA-DR).\textsuperscript{6,7} Preincubation of monocytes with anti–TNF-R1 indicated that TNF-α contributed to VSMC apoptosis by way of monocyte TNF-R1. Although anti–TNF-R2 antibodies inhibited macrophage-induced HCMED1-E6 VSMC apoptosis, only macrophages expressed TNF-R. These findings suggested that macrophage maturation in culture requires autocrine TNF-α. We therefore tested whether antagonistic anti–TNF-R2 and anti–TNF-R1 antibodies modulated macrophage surface Fas-L expression and nitrite efflux, previously described mechanisms of macrophage-induced VSMC apoptosis in monocytes of macrophages. In these cultures, there would be no source of TNF-α but the macrophages themselves.

Monocytes were cultured in isolation for 8 days to allow differentiation to macrophages, in the presence and absence of neutralizing antibodies to TNF-R1 or TNF-R2. Macrophage nitrite efflux was reduced by anti–TNF-R1 or anti–TNF-R2 antibodies (online Figure IIA and IIB; please see http://atvb.ahajournals.org). Incubation of 8-day macrophage monolayers with neutralizing anti–TNF-R1 or with anti–TNF-R2 antibodies also abolished surface Fas-L expression in macrophages cultured in isolation to maturation at day 8 (online Figure IIC). This indicates that macrophage autocrine TNF-α was required for expression of macrophage Fas-L. Thus, macrophage-derived TNF-α acts in an autocrine pathway by way of TNF-R1 and TNF-R2 to promote NO synthesis and Fas-L surface expression. Because TNF-α was required for expression of Fas-L, we also examined whether the converse was true. However, antagonistic anti–Fas-L antibodies had no effect on macrophage TNF-α expression (online Figure IID). Thus, whereas TNF-α controlled Fas-L, Fas-L did not control TNF-α, indicating that Fas-L lies downstream of TNF-α for macrophage cytotoxic activation.

**Macrophage TNF-α Expression Is Dependent on Medium Lipoproteins**

Within the vessel wall, the transition from monocytes to macrophages is accompanied by accumulation of modified lipoproteins, many of which have been implicated in macrophage activation. We therefore examined the role of lipoproteins in TNF-α–associated macrophage activation. We examined TNF-α and Fas-L surface expression, nitrite efflux, and VSMC apoptosis in macrophages cultured in control medium or in medium constituted with lipoprotein-depleted fetal calf serum (LDFCS), as described\textsuperscript{20} (online Figure IIIA–IIIJ; please see http://atvb.ahajournals.org).

Incubation in LDFCS had no effect on macrophage survival (data not shown) but reduced both macrophage-induced plaque VSMC apoptosis (apoptotic index, mean±SEM: control, 72±5.5%; LDFCS, 25±3.4%) and nitrite efflux (control, 438±56 nmol nitrite; LDFCS, 290±20 nmol nitrite; P<0.05). Incubation in LPDFCS consistently reduced the spontaneous expression of surface Fas-L and TNF-α by isolated, cultured macrophages at maturation day 8 (online Figure IIIA and IIIB). Importantly, the addition of physiologic levels of LDL (0.2 mg/mL)\textsuperscript{20} reconstituted the typical expression of surface Fas-L and TNF-α by isolated macrophages at culture day 8 (online Figure IIIC). In addition, the scavenger receptor ligands acetyl-LDL, maleyl–bovine serum albumin, and polyinosine (but not polycytosine, a recognized control for polyinosine\textsuperscript{21}) stimulated macrophage surface Fas-L and TNF-α expression (online Figure IIID–IIIG). Furthermore, an inhibitory antibody to the macrophage scavenger receptor anti-CD36 antibody, clone SM macrophage, reduced macrophage-induced HCMED1-E6 VSMC apoptosis in a concentration-dependent manner (online Figure IIIH). Flow cytometry confirmed that the macrophages expressed CD36 (Figure IIIJ) as before.\textsuperscript{22} Thus, macrophage-induced apoptosis and surface Fas-L expression at culture days 6 to 8 is lipoprotein dependent and scavenger receptor dependent.

**TNF-R1 and TNF-α Are Expressed in Human, Ruptured, Carotid Plaques**

For TNF-α to contribute to macrophage-induced VSMC apoptosis and the rupture of human plaques, then it should be expressed in ruptured human carotid atherosclerotic plaques. Indeed, immunohistochemistry demonstrated that CD68-positive macrophages were immunoreactive for TNF-α, and
α-actin–positive VSMCs were immunoreactive for TNF-R1 (online Figure IV; please see http://atvb.ahajournals.org).

**Discussion**

Macrophage-induced VSMC apoptosis could contribute to plaque rupture.\(^1,2,25\) We have recently reported that human blood–derived macrophages induce apoptosis in human plaque–derived VSMCs in direct coculture, involving direct cell-cell contact, Fas, and NO.\(^6\) In this study, we demonstrate that TNF-α promotes macrophage-induced VSMC apoptosis. Thus, blockade of TNF-R1 and TNF-R2 each inhibited VSMC apoptosis. Western blotting and flow cytometry showed that TNF-R1 and TNF-R2 were expressed by monocytes and VSMCs and that macrophages expressed cell surface TNF-α. TNF-R1 was expressed on the cell surface in plaque VSMCs, and its cell surface expression was induced by NO in HCMED1-E6 VSMCs. Thus, TNF-α and its receptors were appropriately located to effect apoptosis.

However, TNF-α does not act in isolation, as macrophage-induced VSMC apoptosis also requires NO and Fas-L.\(^6\) Data presented here indicate that there are cooperative interactions between TNF-α, Fas-L, and NO, modulating both macrophage activation and VSMC responsiveness. The use by macrophages of TNF-α, NO, and Fas-L to effect apoptosis might appear redundant. However, our experiments have indicated that inhibition of any 1 of these might result in substantial inhibition of apoptosis, even though the other 2 mechanisms are not directly inhibited. This suggests that cooperative interactions might occur between these 3 mediators, and the present study outlines several such interactions.

Fas and TNF-R1 are homologues and initiate caspase-dependent death signaling by similar mechanisms.\(^13,14\) Others have previously found that apoptosis induced by Fas-L and TNF-α is synergistic.\(^14\) We have confirmed that this synergism also applies to cultured, plaque-derived VSMCs. Indeed, this synergism provides further explanation for the apparent requirement for all 3 mediators in macrophage-induced VSMC apoptosis. Here we showed that NO upregulated TNF-R1 on VSMCs, which would be expected to sensitize them to TNF-α–induced apoptosis. We have previously shown that NO similarly upregulates surface Fas\(^7\) and that p53 upregulates surface Fas and TNF-R1.\(^19\) Earlier, Geng et al\(^{11}\) likewise importantly showed that cytokines induce apoptosis of medial VSMCs and sensitize VSMCs to Fas-induced apoptosis.\(^5\) We studied the effects of Fas-L, TNF-α, and NO on plaque-derived VSMCs, which are “primed” for apoptosis. We found with plaque VSMCs that although NO, TNF-α, and Fas-L could induce some apoptosis in isolation, they induced apoptosis synergistically with each other. Thus, not only does NO “gate” VSMC activation for apoptosis by Fas and TNF-α, but also Fas and TNF-α gate each other.

We have previously published data that the monocytes used were >99% pure and free of T lymphocytes.\(^6\) Moreover, we have shown that inclusion of lymphocytes reduces the efficacy of macrophage-induced apoptosis\(^6\) and that macrophage cytotoxic activation in these preparations was positively identified as dependent on lipoproteins. Thus, we are confident that we have excluded macrophage activation via TNF-R due to major histocompatibility complex mismatching and rejection-type phenomena.

However, further interactions are revealed by analysis of the monocyte-macrophage transition. We have previously shown that human peripheral blood monocytes added to culture become activated proapoptotic macrophages expressing activation markers CD16 and HLA-DR.\(^6\) The monocyte-macrophage transition in vivo is often considered analogous to the monocyte-macrophage transition in vivo, although the extent of similarity is not clear. Monocyte-macrophage activation might be stimulated by adhesion in vitro\(^25\) and by transendothelial migration in vivo.\(^25\) We have previously shown that macrophages acquire proapoptotic potential after 5 to 8 days in culture,\(^6\) and others have shown that oxidized lipoproteins stimulate macrophage liberation of soluble TNF-α in vitro.\(^26\) Although the mechanism for this effect was not studied, it is most likely by scavenger receptor ligation, because scavenger receptors are the major receptors for oxidized lipoproteins\(^1\) and activate macrophages.\(^22,27,28\) We have extended this concept to involve spontaneous macrophage maturation in culture.

Our observed downregulation of macrophage TNF-Rs with maturation is consistent with the literature.\(^29–32\) Furthermore, stimulation by TNF-α is recognized to downregulate TNF-R1 and TNF-R2.\(^30,32\) In endothelial cells, NO stimulates shedding of TNF-R1.\(^29\) Thus, loss of TNF-R1 and TNF-R2 could reflect macrophage autocrine stimulation via NO or TNF-α.

Although TNF-R1 and TNF-R2 were required for macrophage-induced VSMC apoptosis in vivo, monocytes expressed TNF-R1 and TNF-R2, raising the possibility that monocytes were a site of action of TNF-α. This was confirmed by preincubation experiments, which showed that incubation of monocyte-macrophage monocultures with anti–TNF-R1 or TNF-R2 antibodies from commencement abrogated macrophage Fas-L and NO in the mature macrophages and inhibited macrophage-induced VSMC apoptosis. Cooperative action of both TNF-R1 and TNF-R2 mediating an effect of TNF-α is well preceded in other systems, eg, in HLA-DR induction in endothelial cells\(^12\) or macrophage activation by CD40.\(^18\)

Although TNF inhibition inhibited Fas-L, the converse was not true—Fas-L inhibition did not inhibit TNF-α surface expression, indicating that Fas-L is downstream of TNF-α. In contrast, NO and TNF-α appear to be mutually dependent, because inhibiting NO reduced TNF-α and vice versa. The reason for this “chicken-and-egg” scenario is uncertain. However, in vivo, macrophages need to respond quickly to challenge. The interdependency could provide a positive-feedback loop, thereby accelerating macrophage activation in response to stimuli from tissue injury or pathogens. Thus, TNF-α promotes macrophage-induced VSMC apoptosis through several interactions, namely: (1) TNF-R1 is synergistic with Fas on VSMCs, (2) NO upregulates VSMC TNF-R1, (3) NO is synergistic with TNF-α to induce VSMC apoptosis, and (4) TNF-α upregulates macrophage inducible NO synthase (iNOS) and surface Fas-L through autocrine TNF-R1 and TNF-R2. Macrophage activation is variably defined. The best accepted markers are CD16 and HLA-DR. We have previously shown that macrophages in our culture system express surface Fas-L, secrete NO, and become proapoptotic in parallel with expression of CD16 and HLA-DR.\(^6\) Our data that anti–TNF-R1 and anti–TNF-R2 antibodies reduce NO
secretion and surface Fas-L indicate that inhibiting autocrine TNF-α reduces macrophage activation. In express and degrades iNOS, cell surface Fas-L, and TNF-α, which in combination induce cell–cell contact–dependent VSMC apoptosis.⑥ Peripheral blood monocytes migrate into atherosclerotic plaques, phagocytose oxidized lipoproteins by scavenger receptors, and become activated to macrophages.① Macrophage differentiation in plaques is associated with expression of Fas-L and iNOS.②③④ If our in vitro system is related to these findings in plaques, then proapoptotic activation should be dependent on culture lipoproteins (which occurs normally in fetal calf serum), and on scavenger receptors, which was confirmed. Furthermore, this model predicts that plaque macrophages should express TNF-α and plaque VSMCs should express TNF-R1, which we also confirmed. In conclusion, we have demonstrated that TNF-α promotes human macrophage–induced VSMC apoptosis by cooperative interactions with NO and Fas-L/Fas.

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Supplementary Online material

Materials and Methods

Isolation of peripheral blood mononuclear cells and subsets and cell culture

Human peripheral blood mononuclear cells (PBMCs) were prepared from buffy coats (East Anglian Blood Transfusion Service), screened for human immunodeficiency, hepatitis B and C viruses. PBMCs were isolated by centrifugation over Ficoll-Hypaque (Histopaque 1077, Sigma) and separated into monocyte-rich and lymphocyte rich fractions as before. Culture flasks were coated with 1% gelatin (Sigma) for 2 hours at 37°C and PBMCs incubated in coated flasks for 1 hour at 37°C. Non-adherent cells were removed with 3 gentle washes of M199 medium and adherent cells removed by brisk flushing with PBS containing 0.5mM EDTA at 4°C. Cell viability was confirmed with trypan blue exclusion and the purity of monocyte or lymphocyte-rich fractions confirmed by flow cytometry as before. The macrophages were >99% pure and contained no T-lymphocytes. After 8 days culture, the macrophage activation was confirmed by expression of CD16 and HLA-DR. The coculture model used has been previously described and validated. Briefly, macrophages and VSMCs were co-cultured and VSMC apoptosis counted by fluorescence microscopy for DNA-binding fluorochromes and fluorescent macrophage marker CD-14-FITC. The mode of death has been demonstrated to be apoptosis with multiple techniques. Cultures were incubated with neutralising anti-TNF-R1 antibody (clone MAB225, R&D Systems, 10µg/ml) neutralising anti-TNF-R2 antibody (clone P80, Genzyme, 1µg/ml) or isotype-matched control antibody (MOPC31, Sigma). Only azide-free antibodies were used in cell cultures and the antibodies were lyophilised and dissolved in PBS pH 7.4. Western blotting, flow cytometry and immunohistochemistry for TNF-R1 and TNF-R2 were by modifications of described methods (Online).
Flow cytometry

Indirect staining was used for flow cytometry of TNF-R1 and TNF-R2 in VSMCs and monocyte/macrophages. Cells were stained as above with unlabelled primary antibody or isotype control (anti-TNF-R1, 10µg/ml Clone MAB225, R&D Systems or 10µg/ml Clone P60, Genzyme; anti-TNF-R2, 10µg/ml clone P80, Genzyme or 10µg/ml Clone utr-1, Bachem; or control IgG1 MOPC31, Sigma), then incubated with fluoresceinated sheep anti-mouse antibody (Sigma) for 30 min at 4°C. Cells were analysed as above. Staining for TNF-α was with a direct method using fluorescein-labelled anti-TNF-α (clone MAB210F, R&D Systems). For comparison of surface and total staining, cells were fixed (4% paraformaldehyde, 15 min 4°C) and then incubated in the presence or absence of 0.1% Triton-X100 at 4°C 15 mins as before.

Human vascular smooth muscle cells (VSMCs).

Human aortic VSMCs (ASMCs) were obtained from donors from Addenbrooke’s Hospital Transplant Programme. Human plaque VSMCs were isolated from carotid plaques, removed at carotid endarterectomy for cerebrovascular disease. Ethical Committee approval for use of discarded tissue for research was obtained. Aortic or carotid plaque tissue was dissected under sterile conditions and explant-cultured in 6-well dishes (Falcon) in M199 medium supplemented with 20% FCS, antibiotics, L-glutamine and amphotericin. Histological examination of specimens confirmed that cells were medial (aortic) or intimal (carotid plaque) in origin. Cells were grown to confluence, and passaged to T75 flasks. Cells were passaged at confluence to approximately passage 7-9 (aortic VSMCs) or passage 1 (plaque VSMCs). The identity of VSMCs was confirmed with α-smooth-muscle-actin immunocytochemistry and morphology in culture. Aortic VSMCs and plaque VSMCs were used after approximately the same time in
Quantitation of apoptosis in macrophage/VSMC co-cultures

VSMCs were seeded in 8-well chamber slides at $10^3$ cells / well (plaque VSMCs, corresponding to $10^3$ cells / cm$^2$) or $2 	imes 10^4$ cells / well (HCME1-E6 cells, corresponding to $2 	imes 10^4$ cells / cm$^2$) and incubated overnight. Macrophages were added at 4:1 macrophages:HCME1-E6 VSMCs ($8 	imes 10^5$ macrophages/well, $8 	imes 10^5$ macrophages/cm$^2$) or 8:1 macrophages:plaque VSMCs ($8 	imes 10^5$ macrophages / well and $8 	imes 10^3$ macrophages / cm$^2$). Apoptosis was quantified by incubation of co-cultures in $10 \mu g/ml$ PI (Sigma) and $125 \mu g/ml$ bisbenzimide (Hoechst 33258; Sigma) for 5 min and incubation with CD-14-FITC to identify macrophages, fixed in 400$\mu$l 10% neutral-buffered formaldehyde, and analysed by fluorescence microscopy. The amount of cell death was measured as the apoptotic index:

$$\text{Apoptotic Index} (%) = \frac{\text{Dead cells}}{\text{(Live Cells + Dead Cells)}} \times 100$$

Macrophages and VSMCs were cocultured for 8 days as before$^6$. Inhibitors were added at the start of co-culture at concentrations of $10 \mu g/ml$ (neutralising anti-TNF-R1 antibody, clone MAB225, R&D Systems; neutralising anti-TNF-R2 antibody, clone P80, Genzyme, or an isotype-matched control antibody, MOPC31, Sigma), or else as detailed (preincubation protocols). These experiments were carried out at the same time and on the same batch of cells as those in a previous paper$^7$. To induce apoptosis in VSMC monocultures, plaque VSMCs were cultured in the presence or absence of $100 nM$ soluble TNF-α (R&D Systems) $1.6 \mu M$ soluble Fas-L (a generous gift from Dr Peter Kiener, Bristol Myers Squibb, NJ) or $100 \mu M$ DETA/NO (Alexis) and apoptosis assessed after 2 days by PI/Hoechst.
Inhibition of macrophage and VSMC death mediators by selective preincubation

Macrophage-induced VSMC apoptosis was identical whether macrophages and VSMCs were co-cultured for 8 days or VSMCs were added to macrophages at macrophage culture day 6. Therefore macrophage and VSMCs were preincubated separately in neutralising antibodies prior to coculture from day 6-8. Apoptosis was assessed as before.

Inhibition of TNF-R1 in macrophages and plaque VSMCs by selective preincubation

Plaque VSMCs were incubated in 10μg/ml neutralising anti-TNF-R1 (MAB225, R&D Systems) or 10μg/ml control antibody MOPC31C for 24 h then trypsinised into suspension and added at 10^3 cells/well to coculture with macrophages. Macrophages (8x10^3 cells/well) were incubated in 10μg/ml neutralising anti-TNF-R1 or 10μg/ml control antibody MOPC31C for 6 days from the start of culture. Both cell types were washed in 3 exchanges of 100 x medium volume prior to coculture to prevent carry-over of unbound antibody. Macrophages and VSMCs were co-cultured for 2 days then VSMC apoptosis assessed with PI/Hoechst/CD14 as before.

Inhibition of TNF-R1 in macrophages and HCMED1-E6 VSMCs by selective preincubation

HCMED1-E6 VSMCs were cultured as before. Blood-monocyte derived macrophages were isolated and cultured as before. HCMED1-E6 VSMCs were incubated trypsinised into suspension and added at 2x10^4 cells/well to coculture with macrophages. Macrophages (8x10^4 cells/well) were incubated in 10μg/ml neutralising anti-TNF-R1 or 10μg/ml control antibody MOPC31C for 6 days from the start of culture. The macrophages were washed x3 in medium prior to addition of the VSMCs. Macrophages and VSMCs were co-cultured for 2 days in 10μg/ml neutralising anti-TNF-R1 (MAB225, R&D Systems) or 10μg/ml control antibody MOPC31C then VSMC apoptosis assessed with PI/Hoechst/CD14 as before.
Western blotting

Non-reducing protein lysates of cultured VSMCs, monocytes or macrophages were prepared as before and 10µg protein/well electrophoresed on a 10% polyacrylamide gel. Proteins were transferred by semi-dry blotting (Biorad) onto PVDF membranes (NEN). Membranes were incubated with 10µg/ml anti-TNF-R1 (clone P60, or 10µg/ml anti-TNF-R2 (clone utr-1, Bachem) in PBST (PBS, 4% non-fat milk protein (Marvel), 0.05% poly-oxy-ethyl-sorbitan-monolaureate (Tween 20)) at 4°C for 18 hours, washed 3x1 min and 4x5 min in PBST, and incubated with sheep-anti-mouse-peroxidase conjugated antibodies (Amersham) in PBST for 2 hours. Membranes were washed 3x30s and 4x5 min and visualised with enhanced chemiluminescence (ECL, Amersham).
**Lipoproteins and macrophage activation**

Lipoprotein-depleted fetal-calf-serum (LDFCS) and human Low-Density-Lipoprotein (LDL) were the gift of Dr C. Fitzsimmons, Cambridge University, prepared by the established ultracentrifugal procedures. Control sera were from the same batch of fetal calf serum. Sera were added to a final concentration of 20% in M199. Acetylated-LDL and maleylated-bovine serum albumin were the kind gifts of Dr D Proudfoot, Dept of Medicine, Cambridge University and was added to cultures at 200µg/ml. Polyinosinic acid and Polycytidylic acid (Sigma) were added to cultures at 100µg/ml.

**Immunohistochemistry**

Endarterectomy specimens of ruptured carotid plaques were fixed in formalin, processed to paraffin and serially sectioned. Consent for use of tissues for research, Addenbrooke’s Hospital Local Research Ethics Committee and Hammersmith Hospitals Local Research Committee approval for the use of tissues in research were obtained. Sections were de-waxed, incubated in sheep polyclonal anti-TNF-α (The Binding Site) or mouse monoclonal anti-TNF-R1 (Genzyme), and visualised with indirect avidin-biotin-immunoperoxidase (Dako) according to manufacturers instruction. Negative controls were pre-immune rabbit serum (Dako) or mouse monoclonal IgG1 MOPC31C (Sigma). To identify cell types, sections were incubated in monoclonal anti-CD68 (clone KP-1, Dako) or anti-α-smooth-muscle-actin (clone α-1-A4, Sigma) and visualised with rabbit-anti-mouse alkaline phosphatase/Vector Blue.
Statistical methods
Statistical analysis used Statview on a PC-microcomputer as before. Binomially distributed data were analysed by analysis of variance (ANOVA) and skewed data were analysed with Dunn’s test (a modification of Kruskal-Wallis) as before. The level of significance was adjusted for multiple simultaneous comparisons.
Online Figure Legends

Figure I Co-operativity between TNF-α, Fas-L and NO to induce VSMC apoptosis.
DETA/NO or DETA were added to cultures of (A) HCMED1-E6 VSMCs or (B) Aortic medial VSMCs and surface TNF-R1 detected after 24 hours n = 5 experiments. (C)(D) NO-donor NO-donor DETA/NO sensitises carotid plaque VSMCs to apoptosis induced by TNF-α. Carotid plaque VSMCs were incubated with TNF in addition to either DETA/NO (C) or Fas-L (D) and apoptotic index estimated at 48 hours. Data are mean ± S.E.M., n=5. * P<0.05, ANOVA.

Figure II
The effect of antagonistic antibodies to TNF-R1 (A) or TNF-R2 (B) on macrophage nitrite efflux. Macrophages were incubated in the presence of absence of neutralising anti-TNF-R1 (10µg/ml MAB225) or TNF-R2 (1µg/ml P80) for 8 days, or IgG isotype-controls. Macrophage-derived nitrite was measured as an index of NO-secretion.

Macrophage nitrite efflux was reduced by anti-TNF-R1 (untreated controls 391±30, IgG controls 385±20, anti-TNF-R1 169±22 pmol nitrite/cell, p<0.05, Student’s t-test, n=50 experiments for n=5 macrophage donors) or anti-TNF-R2 antibodies (untreated controls 248±20; IgG controls 248±40; anti-TNF-R2 174±27 pmol nitrite/cell, Student’s t-test, p<0.05, n=50 experiments for n=5 macrophage donors). Because the effects of antibodies to TNF-R1 and to TNF-R2 were tested as different experiments, the data were not pooled.

(C) Effect of antagonistic antibodies to TNF-R1 and TNF-R2 on macrophage surface Fas-L macrophage cultures were treated for 8 days with control, antagonistic anti-TNF-R1 or anti-TNF-R2 antibodies and surface Fas-L detected by flow cytometry (n = 5 experiments).

(D) Effect of antagonistic antibodies to Fas-L on macrophage surface TNF-α. Macrophages were incubated with antagonistic anti-Fas-L antibodies for 8 days and stained for surface TNF-α (n = 5).
Figure III Effect on medium lipoproteins on macrophage surface expression of Fas-L and TNF-α.

(A-G) Macrophages were incubated for 6 days in control serum, lipoprotein-depleted serum (LPDFCS) or LPDFCS reconstituted with LDL or scavenger receptor ligands LDL, acetyl-LDL, polyinosinic acid (Poly-I) and maleyl-BSA, and surface Fas-L detected at 24 hours. Polycytidylic acid (Poly-C) is a negative control for Poly-I. n=5-10 experiments for n=5-10 macrophage donors.

(H) Human peripheral blood macrophages were incubated for 6 days in the presence and absence of antagonistic anti-CD36 antibodies at the concentrations indicated (1, 3, 10 µg/ml). At culture day 6 HCMED1-E6 VSMCs were added and after 48 hours apoptotic index measured. * p<0.05, compared to control cocultures. Student’s t-test, p<0.05.

(J) Macrophage cultures were stained with anti-CD36 antibodies (Methods). Histogram format as Figure 2.

Figure IV Immunohistochemistry of human carotid plaques for TNF-α and TNF-R1.

Ruptured carotid plaques were double-immunostained for TNF-R1 and alpha-smooth muscle actin (A) or TNF-α and macrophage marker CD68 (B). There is colocalisation of TNF-R1 with smooth muscle actin and of TNF-α with CD68, indicating that macrophages express TNF-α and VSMCs express TNF-R1. Green filled arrowheads, cholesterol clefts. Unfilled arrows, macrophages.
Figure I

(A) TNFR1

(B) TNFR1

(C) Plaque VSMC apoptosis (%)

(D) Plaque VSMC Apoptosis (%)

Control
NO
TNF
TNF+NO

Control
Fas-L
TNF
Fas-L + TNF
Figure II

(A) Surface Fas-L Staining
- Control
- Anti-TNF-R1
- IgG

(B) Macrophage Nitrite Release (pmoles)
- Untreated
- Anti-TNF-R2
- Control IgG

(C) Surface TNF-α Staining
- Control (IgG incubated)
- Anti-TNF-R1 incubated
- Anti-TNF-R2 incubated

(D) Surface TNF-α Staining
- Control Macrophages (IgG incubated)
- Anti-Fas-L Incubated Macrophages
Figure III

(A) CONTROL

(B) LDFCS

(C) LDFCS + LDL

(D) LDFCS + AcLDL

(E) LDFCS + MalBSA

(F) LDFCS + Poly-I

(G) LDFCS + Poly-C

(H) VSMC Apoptosis (%)

Control Coculture
Anti-CD36 - 1
Anti-CD36 - 3
Anti-CD36 - 10

Culture Day 1  Culture Day 8

(J) Anti-CD36 Staining
Figure IV

(A) Negative TNF-α Negative CD68

(B) Negative TNF-α Positive CD68