Release of tumour necrosis factor α (TNFα) by TNFα cleaving enzyme (TACE) in response to septic stimuli in vitro

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Background. Tumour necrosis factor α (TNFα), in its soluble form (solTNF), has been well described as an important cytokine in inflammatory states including sepsis. The transmembrane precursor of solTNF, membrane-bound TNFα (memTNF), is cleaved by TNFα cleaving enzyme (TACE), the regulation of which is poorly understood. We hypothesized that the diversity of clinical features seen with sepsis caused by different organisms may be a result of differential regulation of TACE. Therefore, we measured these proteins in models of sepsis using flow cytometric methods that we have developed.

Methods. Surface protein expression of memTNF and TACE, and TACE catalytic activity were measured in human monocytes by flow cytometry following cell stimulation by lipopolysaccharide (LPS), zymosan (a yeast cell wall product) or heat-inactivated Neisseria meningitidis.

Results. Unstimulated human monocytes express memTNF on the cell surface (mean fluorescence intensity, MFI 131) and this is down-regulated initially in response to LPS (MFI 57) but then recovers to exceed the resting protein expression (MFI 614). TACE protein is also present on the surface of resting cells (MFI 389) but is catalytically inactive until cell stimulation. Stimulation of monocytes with LPS, zymosan and Neisseria meningitidis produced different patterns of TACE activation with time.

Conclusions. The regulation of memTNF by TACE on monocytes is an important regulatory event in the pro-inflammatory cytokine cascade. As monocytes are important in the inflammatory cascade, we suggest that the control of memTNF and TACE activity on monocytes may play a role in the pathophysiology of sepsis.

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Much published work has focused on the role of soluble tumour necrosis factor α (solTNF) in mediating both acute and chronic inflammatory diseases. Membrane-bound TNFα (memTNF) has been considered as only a transient precursor. However, there is now increasing evidence that memTNF is itself a biologically relevant molecule.

The importance of TNFα in the pathogenesis of septic shock has been well described. Trials involving the administration of low doses of endotoxin to healthy volunteers resulted in the transient occurrence of a sepsis-like syndrome and elevated TNFα levels were measured in these volunteers.1,2 In a paediatric population with septic shock, procalcitonin, IL-10, and TNFα were associated with the severity of organ failure and mortality.3 However, anti-TNFα antibodies have had disappointing results when used in clinical trials for patients with sepsis.4,5 All these studies have concentrated on the role of solTNF alone.

Human TNFα is initially expressed as a 26 kDa non-glycosylated type II transmembrane protein (memTNF). MemTNF undergoes cleavage by TNFα convertase enzyme (TACE) on the cell surface to release a 17 kDa soluble protein (solTNF), which exists as a homotrimer.6 TACE is a membrane-bound enzyme that is a member of the family of ADAMs (a disintegrin and metalloproteinase). The importance of TACE in the control of TNFα release lies not solely in the production of a circulating soluble factor (solTNF),

1Part of this paper has been presented in abstract form at the ARS meetings in Nottingham (November 2001) and Cardiff (July 2002).
but also in the removal of \textit{mem} TNF, which has its own biological effects and the TACE-dependent cleavage of the p55 TNF receptor, as once cleaved this receptor neutralizes the effects of released solTNF.

Early work has shown that when a non-cleavable TNF\textalpha mutant is made (by site-directed mutagenesis), this \textit{mem} TNF, which cannot be converted to the circulating soluble factor, is capable of killing tumour and virus-infected cells through cell-to-cell contact. Transgenic mice that produce non-cleavable TNF are protected from endotoxic shock but may have more organ-specific diseases such as arthritis and hepatitis. These studies indicate that \textit{mem} TNF exerts distinct functions from solTNF in the induction of cell death (through signaling via the p75 TNF receptor) and production of organ-specific inflammation in animal models. However, results from such models are limited in their interpretation as \textit{mem} TNF is over-expressed to such a large extent that many regulatory mechanisms in the cell may be affected.

The cleavage of \textit{mem} TNF to solTNF is regulated by TACE but investigations into the regulation of TACE protein expression and catalytic activity have been limited. Doedens and Black studied the regulation of TACE catalytic activity by examining the effects of cell stimulation on TACE localization and expression. Immunofluorescence microscopy revealed a punctate distribution of TACE on the surface of untreated cells and their experiments suggested that cell-surface TACE was internalized in response to stimulation with phorbol 12-myristate 13-acetate. This work was performed using confocal microscopy and TACE activity was not examined. A quenched fluorescent substrate assay has been used to assess the activity of other matrix metalloproteases in cell-based assays but this has not been suitable for assessing TACE activity because of the lack of absolute specificity of the substrate for cleavage by TACE.

The aims of this study were to examine the kinetics of \textit{mem} TNF, TACE surface protein, and catalytic activity using methods we have developed in our own institute. The regulation of \textit{mem} TNF and TACE on human monocytes was studied following stimulation of monocytes with molecules relevant to the initiation of sepsis in order to understand the regulation of \textit{mem} TNF and TACE in acute inflammatory states. This work investigated the hypothesis that regulation of the cleavage of \textit{mem} TNF and the consequent release of solTNF by TACE on monocytes may be a factor in the pathophysiology of sepsis.

Materials and methods

\textbf{Materials}

All reagents and consumables were purchased from Sigma Chemical Company, Poole, Dorset, UK, unless otherwise stated. Ficoll/hypaque lymphoprep (specific gravity 1.077 g ml\textsuperscript{-1}) was purchased from Axis-Shield PoC AS, Oslo, Norway, anti-CD14, anti-CD45, anti-CD3, and anti-CD19 monoclonal antibodies from Becton Dickinson, Oxford, UK. Anti-human TACE murine monoclonal antibody (M222) was kindly provided by Dr R. Black, Immunex, WA, USA and the F(ab\textsuperscript{'}\textsuperscript{2}) fraction of an anti-human TNF\textalpha chimeric antibody (cA\textsubscript{2}) and the biotinylated anti-mouse idiotpe M101 from Dr J. Ghrayeb, Centocor, Malvern, PE, USA. Goat anti-mouse Ig glycoerythrin (PE) and streptavidin PE were purchased from Southern Biotech, Birmingham, AL, USA. Capture antibody (purified mouse anti-human TNF\textalpha) and detection antibody (biotin mouse anti-human TNF\textalpha) for sandwich ELISA were from Pharmingen, San Diego, CA, USA. Peroxidase substrate system for sandwich ELISA was purchased from Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA. BB94 (Roche, East Sussex, UK), a peptidic hydroxamate inhibitor, was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 \mu M where DMSO is less than 1% into final solution. Heat-inactivated Neisseria meningitidis (10\textsuperscript{6} N. meningitidis cells per 10\textsuperscript{6} monocytes) was a kind gift of the Medical Microbiology Department, St Mary’s Hospital, London, UK.

\textbf{Cells and cell culture}

Human monocytes were separated and purified by centrifugal elutriation from a single donor platelet-depleted residue (North London Blood Transfusion Service, Colindale, UK) as described previously. Monocytes separated by this method were routinely more than 80% CD14 expressing cells with less than 3% CD3 expressing cells as assessed by flow cytometry (FACScan, Becton Dickinson, Oxford, UK).

\textbf{Cell stimulation and harvest}

Separated monocytes were plated at a density of 1\times 10\textsuperscript{6} cells per well in 1 ml in a 24-well plate. Monocytes were stimulated with lipopolysaccharide (LPS Salmonella typhimurium 10 ng ml\textsuperscript{-1}), zymosan (30 \mu g ml\textsuperscript{-1}) or N. meningitidis (10\textsuperscript{6} organisms per 10\textsuperscript{6} monocytes). These stimuli were chosen to reflect the variety of infective agents with different mechanisms of action in clinical practice and the concentrations used gave the maximum sol TNF release at 4 h in dose–response experiments (data not shown). Following cell stimulation, the plates were placed in an incubator at 37\textdegree C with 90% humidity and carbon dioxide 5%/air 95%. At the time of harvest the cells were transferred to FACS tubes and centrifuged at 180 relative centrifugal force (RCF) for 5 min. The supernatant was removed for analysis of solTNF\textalpha by sandwich ELISA. Remaining cells were fixed using 100 \mu l of 1% paraformaldehyde on ice for 10 min, then washed using 500 \mu l of FACS wash buffer (FWB) (PBS containing 2% fetal calf serum and 0.02% sodium azide) and centrifuged at 1200 RCF for 5 min. The resulting cells were then stained with appropriate antibodies or isotype controls.
**Determination of memTNF and TACE by flow cytometry**

For detection of surface TACE protein, cells were treated with M222 (1 μg ml\(^{-1}\)) then 250 ng ml\(^{-1}\) of goat anti-mouse Ig PE. MemTNF was detected using 10 μg ml\(^{-1}\) of c\(_2\)F(ab\(^{-})\)_2, 5 μg ml\(^{-1}\) of biotinylated M101 then 250 ng ml\(^{-1}\) of streptavidin PE. For each protein of interest, cells were incubated with 50 μl of the appropriate antibody for 30 min on ice. After this and all subsequent steps, the cells were washed with 500 μl of FWB. Flow cytometric analysis (FACS) was performed using a FACScan (Becton Dickinson, UK). The results were calculated from 10 000 acquired cells for each of seven independent experiments and expressed as mean fluorescence intensity (MFI).

**Determination of solTNF by sandwich ELISA**

SolTNF was determined by sandwich ELISA (according to manufacturer’s instructions) in seven independent experiments. The absorbance of the plates was read at A\(_{450\text{nm}}\) on a plate reader using a Delta Soft II.4 software programme. Results, based on triplicate cultures, are expressed as mean concentration (pg ml\(^{-1}\)) (1 SD).

**Determination of TNF\(\alpha\) bioactivity by WEHI assay**

WEHI 164 mouse monocyteic cell line (clone 13)\(^{15}\) was grown in DMEM with 5% FCS until confluent and TNF\(\alpha\) bioactivity determined as described previously.\(^{15}\) The absorbance of the blue formazan product was read at A\(_{620\text{nm}}\).

**Confocal microscopy**

Glass coverslips were coated with 500 μl of gelatin and incubated at room temperature for 10 min. Excess gelatin was then removed and the coverslips sequentially incubated with 500 μl of each of 1% gluteraldehyde, 1 M ammonium chloride and 70% ethanol. After three washes with RPMI containing 5% FCS, the cells were added to the gelatin-coated coverslips and incubated for 2 h at room temperature until they had adhered. Excess media was removed and 500 μl of 3% paraformaldehyde added for 10 min to fix the cells and then 500 μl of 3% BSA for 1 h to block coverslips (with the coverslips washed twice with 1 ml of PBS between each step). In some experiments the cells were permeabilized by the addition of Triton X-100 (0.1% in PBS) for 5 min before the addition of 3% BSA. The primary antibody was added (M222 at 1 μg ml\(^{-1}\)) and following a further two washes with 1 ml of PBS, a secondary antibody was added (goat anti-mouse IgG conjugated with Alexa 488 at 10 μg ml\(^{-1}\)). The coverslips were then transferred to glass microscopy slides and visualized using confocal microscopy.

**Statistical analysis**

Statistical analysis was performed using Prism 3.0 software. Flow cytometric data are presented as mean fluorescence intensity (MFI [1 SD]) of 10 000 counted cells with the relevant isotype control subtracted. ELISA data are shown as mean concentration (pg ml\(^{-1}\)) (1 SD) and, as the data were normally distributed, a one-way ANOVA test was applied where P<0.05 was considered to be statistically significant.

**Results**

**Measurement of memTNF**

To detect memTNF by flow cytometry we used an optimized combination of antibodies. Time-dependent changes in expression of memTNF were examined in elutriated monocytes stimulated with LPS (10 ng ml\(^{-1}\)) (Fig. 1A). Constitutive memTNF was detected on the surface of resting monocytes. This decreased after 1 h of stimulation but returned to resting values by 2 h. MemTNF expression continued to increase up to 36 h following stimulation. These changes in memTNF were inversely related to solTNF (Fig. 1B). Thus, solTNF levels from stimulated, elutriated monocytes were detectable after 1 h and were maximal by 2 h.
To confirm that the memTNF measured by flow cytometry with this antibody combination was biologically active, we used a WEHI assay (Fig. 2). Unstimulated monocytes had no killing effect on the WEHI cells indicating that there was no biologically active memTNF on unstimulated cells. With LPS stimulation of monocytes there was an increase in cell death, which was further augmented by the addition of BB94 throughout the culture period to inhibit all proteases, including TACE. This cell death directly correlated with the increase in memTNF detected by flow cytometry. The antibody cA2 has been well described to neutralize TNFα (in vitro and in vivo) and the addition of neutralizing cA2 to the cultured monocytes inhibited the activity of memTNF, reflected in the WEHI assay by an absence of cell death.

Measurement of TACE surface protein
Surface TACE expression was examined using a flow cytometric method and confirmed by confocal microscopy. TACE is detectable on resting monocytes and, following cell activation, expression decreased in a time-dependent manner with TACE surface protein virtually undetectable 36 h after stimulation (Fig. 3A).

In addition, TACE surface protein expression was examined by confocal microscopy (Fig. 3A). Using the same primary antibody (M222) as for the flow cytometry, TACE is present on unstimulated monocytes and the cell-staining pattern shows that it is localized to the cell surface. Following LPS stimulation, there was no TACE protein detected on the cell surface.

Measurement of TACE catalytic activity
Having established a method to measure surface TACE protein expression by flow cytometry, we proceeded to examine the catalytic activity of TACE. Fluorescent assays for metalloproteases, including recombinant TACE, have been developed but these methods are not easily adapted for assessment of cell-associated TACE activity. Therefore, we investigated whether TACE activity could be measured indirectly using the hydroxamate compound, BB94. Cells were stimulated with LPS, harvested at different time points and then, at harvest, treated with or without BB94 (which blocks all metalloproteases including TACE). MemTNF expression was determined and the fold difference between cells treated with or without BB94 used as an indirect indication of TACE catalytic activity (Fig. 4A). Using this indirect method, TACE activity was undetectable in resting cells, was maximal 2 h following stimulation (14-fold increase) and declined thereafter. This assay showed that, although TACE protein was present on the surface of unstimulated monocytes, it was catalytically...
inactive but rapidly activated 2 h after LPS stimulation. Thereafter, the activity of TACE declined and returned towards unstimulated levels by 36 h.

**memTNF and TACE kinetics were stimulus dependent**

We compared the kinetics of *mem*TNF, *sol*TNF, and TACE catalytic activity in response to stimulation by LPS, with zymosan and with heat-inactivated *N. meningitidis* in elutriated monocytes. As shown previously with LPS stimulation (Fig. 1A), maximal production of *sol*TNF was found 2–4 h after addition of all stimuli, with zymosan the most efficacious stimulus for *sol*TNF release producing a 1.4- and 1.6-fold increase in *mem*TNF at 4 h over LPS and *N. meningitidis* stimulation, respectively (Fig. 5A).

Differential effect of these stimuli on *mem*TNF was assessed by flow cytometry. All stimuli induced an initial (2–4 h) down-regulation in *mem*TNF, which was followed by a gradual increase up to 24 h (Fig. 5B). Whilst the levels of *mem*TNF increased with LPS and *N. meningitidis* (7- and 5-fold, respectively), stimulation with zymosan produced only a 2-fold increase in *mem*TNF. This may indicate continued activation and/or production of TACE in response to zymosan stimulation.

To examine these possibilities, TACE protein was measured in monocytes following addition of these stimuli. All stimuli resulted in a rapid down-regulation in TACE by 2–4 h. Zymosan was the most efficacious stimulus (producing an 8-fold reduction from resting levels) (Fig. 6A) adding to the evidence that zymosan was a more efficacious activator of TACE than the other two stimuli.

To further examine the effects of these stimuli on TACE catalytic activity, we measured TACE activity indirectly using the addition of BB94 at the time of harvest as described previously. Changes in *mem*TNF with the addition of BB94, as an indirect assessment of TACE activity, are shown in Figure 6B. TACE catalytic activity increased at 2 h, and reached a maximum by 4 h after all stimuli. Again zymosan was the most efficacious stimulus for TACE activation in monocytes with a 22–34-fold increase in activity by 2–4 h compared with LPS (12–19-fold increase by 2–4 h) and *N. meningitidis* (8–15-fold increase by 2–4 h).

**Discussion**

We have established methods to measure *mem*TNF and TACE surface proteins and TACE catalytic activity by flow cytometry and examined the effects of various septic stimuli on these proteins *in vitro*. Previous work has focused
on measurement of the concentrations, biological effects, and activity of solTNF alone.

We examined the expression of memTNF on resting and stimulated monocytes with this flow cytometric assay. Unexpectedly, memTNF was detected on the surface of resting monocytes but rapidly decreased (<1 h) following cell stimulation before increasing again to exceed resting values (>24 h). This finding suggests that haemopoietic cells express a measurable amount of biologically active memTNF.

These studies suggest that the gradual latent accumulation of memTNF represented a balance between a time-dependent increase in memTNF synthesis, and a decrease in TACE activity. The notion that there is increased synthesis of memTNF is not supported in these experiments because at 1 h, when memTNF has declined, there is no accompanying increase in solTNF. In contrast, indirect assessment of TACE catalytic activity indicated that TACE bioactivity was maximal at 2 h decreasing thereafter, suggesting that the gradual increase in memTNF may represent a balance between de novo synthesis of memTNF and/or TACE, and TACE catalytic activity.

Surface TACE protein expression was detected using a monoclonal antibody and analysed by flow cytometry. We found that the levels of TACE protein decreased in response to cell stimulation in a time-dependent manner. These findings confirm work by Black and colleagues who demonstrated, using confocal microscopy in THP-1 cells, that surface TACE protein was down-regulated in response to phorbol ester, that down-regulation was a result of internalization of the protein and this only occurred when the enzyme was in the active state. Interestingly, our flow cytometric results suggested that expression of TACE protein on a cell is inversely proportional to its biological activity. Thus, membrane TACE protein is expressed on resting cells, but is rapidly lost from the surface when enzymatic activity is maximal.

The regulation of TACE is an active area of research. Whilst cell stimuli, such as phorbol ester, have been demonstrated to increase TACE gene expression, protein production and furin cleavage of the pro-domain to active TACE, little has been published previously regarding the regulation of TACE catalytic activity as a result of the absence of a suitable activity assay for cell-based models. We confirmed that the regulation of TACE is stimulus-specific using more physiological stimuli. Zymosan was the most efficacious stimulus for solTNF release and resulted in the greatest down-regulation of surface TACE following stimulation. These results indicate that zymosan is a potent activator of TACE catalytic activity in monocytes and may be an alternative stimulus to LPS for models of sepsis.

The differences in memTNF and TACE following these stimuli may reflect the differing signalling pathways involved. It is well known that LPS signals via a receptor complex with cell surface components including CD14 and toll-like receptor-4 (TLR4). There is increasing evidence that the TLR4-centred LPS receptor complex is different stimuli may reflect the differing signalling pathways involved. Bacterial cell components also signal through TLR2 and it has been observed in HeLa cells that both TLR1 and TLR2 are required for the cells to respond to N. meningitidis. Further, Imler and Hoffmann have hypothesized that the ratios of expression of different TLRs on a particular cell may affect the response of that cell to a particular stimulus.

In conclusion, we examined memTNF and TACE surface protein and TACE catalytic activity in a number of in vitro models of sepsis. We have shown that these proteins, and their regulation, vary with septic stimulus used and should be considered significant contributors to the pro-inflammatory cytokine network. In particular, regulation of the cleavage of TNFα by TACE may have important consequences for understanding the pathophysiology of sepsis and requires further in vivo studies.

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