

Continuous real-time measurement of tumor necrosis factor- α converting enzyme activity on live cells

Montserrat Alvarez-Iglesias¹, Gareth Wayne², Kieran P O'Dea¹, Augustin Amour² and Masao Takata¹

¹Department of Anaesthetics and Intensive Care, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, UK and ²Assay Development and Compound Profiling, GlaxoSmithKline, Stevenage, UK

Tumor necrosis factor- α (TNF) converting enzyme (TACE) is responsible for shedding of various membrane proteins including proinflammatory cytokine TNF. *In vivo* regulation of TACE is poorly understood mainly due to lack of reliable methodology to measure TACE activity in cell-based assays. Here we report a novel enzyme assay that enables continuous real-time measurement of TACE activity on the surface of live cells. Cells were incubated with a new fluorescent resonance energy transfer peptide consisting of a TACE-sensitive TNF sequence and fluorescein–tetramethylrhodamine (FAM–TAMRA), and enzyme activity was monitored by the rate of increase in fluorescent signal due to peptide cleavage. Validation studies using resting as well as stimulated monocytic cells indicated that the assay was sensitive, reproducible and quantitative. Pharmacological studies with various inhibitors indicated that the observed enzyme activity could largely be ascribed to TACE. Thus, the FAM–TAMRA peptide provides a powerful tool for measurement of constitutive and inducible cellular TACE activity. The principles developed may be applied to analyses of enzyme activity of various sheddases on live cells.

Laboratory Investigation (2005) **85**, 1440–1448. doi:10.1038/labinvest.3700340; published online 22 August 2005

Keywords: ADAM; ectodomain shedding; enzyme activity; FRET; protease; TACE; TNF

Ectodomain shedding of membrane proteins is recognized as a potent mechanism for downregulation of their cell-associated activity while at the same time enabling their function as soluble mediators.^{1,2} Tumor necrosis factor- α (TNF) converting enzyme (TACE), a member of the ADAM (a disintegrin and metalloprotease) family of proteases (ADAM-17), is the first discovered mammalian sheddase, responsible for cleavage of a variety of membrane proteins, including the proinflammatory cytokine TNF, transforming growth factor- α , p75 TNF receptor and L-selectin.^{3–8} As a TNF sheddase, TACE regulates the *in vivo* cleavage of membrane-bound proTNF to release soluble TNF,^{3,4} which has been shown to play a crucial role in acute and chronic inflammation. Thus, analysis of TACE activity should provide important insights into the

pathophysiology of inflammatory diseases and potentially help the future development of TACE-targeted therapies. However, the mechanism of *in vivo* regulation of TACE activity is not well understood,^{7–9} mainly due to lack of accurate methods for measuring TACE activity on viable cells.

Fluorescence resonance energy transfer (FRET) peptides provide useful tools for kinetic studies of metalloproteases in solution.^{10,11} These substrates consist of a donor fluorophore and a light-absorbing acceptor, attached to the terminal residues of a peptide susceptible to cleavage by a protease. Once the substrate is cleaved, increased fluorescence is observed due to loss of internal quenching, allowing quantitative measurement of real-time enzyme activity. The common donor/acceptor pairs are 4-(dimethylaminoazo) benzene-4-carboxyl (Dabcyl) and 5-(2-aminoethylamino)-1-naphthalenesulfonic (Edans), and (7-methoxycoumarin-4-yl) acetyl (Mca) and 2,4-dinitrophenyl (Dnp) or 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl (Dpa), and peptides containing these pairs have been used for *in vitro* assays of recombinant or purified native TACE.^{12–14} However, application of these FRET

Correspondence: Dr M Takata, MD, PhD, Department of Anaesthetics and Intensive Care, Imperial College London, Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, UK. E-mail: m.takata@imperial.ac.uk

Received 21 March 2005; revised 7 July 2005; accepted 9 July 2005; published online 22 August 2005

peptides to *in vivo* assays with intact cells, that is, continuous real-time measurement of TACE activity on the surface of live cells, has not gained much success,¹⁵ because of insufficient sensitivity and non-specific peptide cleavage in the cellular environment. Doedens *et al*¹⁶ succeeded in detecting cleavage of a Dnp-based peptide by activated mouse monocytic cells, yet in that study substrate hydrolysis was analyzed by high-performance liquid chromatography (HPLC), which precludes direct continuous measurement and is more labor intensive and less quantitative than FRET peptide assays.

Unlike the above fluorophores, fluorescein (FAM) is less susceptible to signal interference due to longer excitation wavelength, large absorption coefficient and quantum yield in the visible region, where output from xenon lamps in fluorimeters is relatively high.¹⁷ These properties allow greater assay accuracy and sensitivity, which may enable signal detection even in the cellular environment. Furthermore, the FAM-tetramethylrhodamine (TAMRA) pair has considerably longer Förster radius (distance at which 50% of internal quenching occurs), allowing longer peptides (14 amino acids vs 9–10 for Dabcyl–Edans or Mca–Dnp pairs) between donor and acceptor¹⁷ and leading to improved specificity. The larger size of FAM and TAMRA may also constrain substrate cleavage by unrelated enzymes.

Here we investigate the application of a new TNF-based FRET peptide with the FAM–TAMRA fluorophore pair for direct continuous measurement of cell-associated TACE activity in live cells. The peptide was first validated *in vitro* with recombinant enzymes, and a cell-based assay was then developed using resting as well as stimulated human monocytic cell lines. The results indicated that the FAM–TAMRA peptide is useful for real-time assessment of TACE activity on the surface of live cells.

Materials and methods

Materials and Synthesis of Peptide

Unless otherwise stated, all reagents were from Sigma (Poole, UK). We synthesized a 13 amino-acid peptide FAM–SPLAQAVRSSSRK–TAMRA (FAM–TAMRA peptide) corresponding to the human proTNF sequence surrounding the TACE-specific cleaving site (Ala–Val bond).⁸ The peptide was synthesized on an *N*^ε-(9fluorenyl)methoxycarbonyl (Fmoc)-amide resin (0.1 mmol) using an ABI 431A peptide synthesizer (Applied Biosystems, Warrington, UK). Fmoc chemistry with *t*-butyl based side-chain protection was used throughout and a 10-fold excess of amino acid was applied at each acylation step. After removal of the Fmoc group with 20% piperidine in dymethylformamide, *N*-terminal acylation with 5-fluorescein carboxylic acid was carried out via 1-hydroxybenzotriazole/diisopropylcarbo-

dimide-mediated coupling. Deprotection and resin cleavage was carried out for 3 h. The crude product was isolated and purified by reversed phase-HPLC using a Jupiter C18 analytical column (Phenomenex, Macclesfield, UK), and fractions containing the FAM-peptide were pooled and lyophilized to constant weight. Reaction with the mixed isomers (5,6-) of tetramethylrhodamine carboxylic acid hydroxy-succinimide ester in 50% dymethylformamide/water/sodium hydrogen carbonate, gave the crude FAM–TAMRA peptide, which was purified and resolved into the two separate isomers by reversed phase-HPLC.

Analysis of FAM–TAMRA Peptide Cleavage by Recombinant Enzymes

Human recombinant (r) TACE or murine rADAM-10 (R&D Systems, Abingdon, UK) were incubated with the FAM–TAMRA TNF peptide over 60 min at 20°C in 20 μ l of tris-based assay buffer (50 mM pH 8.0 Bis-Tris propane, 100 mM NaCl, 10 mM CaCl₂ and 0.01% Triton X-100), using black NUNC polystyrene 384-well microtiter plates (VWR, Poole, UK). For inhibitor studies, a hydroxamate derivative GM6001 (Chemicon Europe Ltd, Hampshire, UK) was used. Enzyme activity measurements were performed by continuous monitoring of peptide cleavage, that is, real-time increases in fluorescence intensity, at an excitation wavelength (λ_{ex}) of 485 nm and emission wavelength (λ_{em}) of 535 nm, using an Ultra spectrofluorimeter (Tecan, Reading, UK). Kinetic analyses were performed with GraFit 3.0 software (Erithacus Software Ltd, Staines, UK). Initial velocities (*V*) of the substrate hydrolysis were determined at various substrate concentrations [*S*] (0–50 μ M), and Michaelis constant (*K*_m) and maximum velocity (*V*_{max}) was estimated by fitting *V* to the equation,

$$V = \frac{V_{\text{max}}[S]}{[S] + K_m}$$

Specificity constant *k*_{cat}/*K*_m values were estimated by linear regression of *V* at low concentrations of [*S*] (< *K*_m) to the simplified Michaelis equation,

$$V = \frac{k_{\text{cat}}}{K_m} [E][S]$$

where [*E*] represents the concentration of the enzyme used.

Comparison of FAM–TAMRA Peptide with other TNF FRET Peptides

Human rTACE was incubated with one of the three peptides, that is, FAM–TAMRA TNF peptide, Dabcyl–Edans TNF peptide (Dabcyl–LA-QAVRSSSR–Edans, purchased from Bachem, St Helen's, UK) or Mca–Dpa TNF peptide (Mca–PLAQAV(Dpa)RSSSR–NH₂, purchased from Calbiochem, Nottingham, UK) at 20°C in the tris-based

assay buffer overnight, until complete cleavage of each peptide was achieved. The reaction products, together with the original uncleaved FRET peptides, were then diluted in either the tris-based assay buffer or CO₂-independent medium (Invitrogen, Paisley, UK), and fluorescent signals measured using a Safire monochromator (Tecan, Reading, UK) at λ_{ex} 485 nm and λ_{em} 535 nm for FAM-TAMRA, λ_{ex} 360 nm and λ_{em} 485 nm for Dabcyl-Edans, and λ_{ex} 320 nm and λ_{em} 400 nm for Mca-Dpa peptides. The ratio of fluorescence intensity between cleaved and uncleaved peptide was calculated for each FRET peptide in each of the assay solutions.

Analysis of FAM-TAMRA Peptide Cleavage by Intact Cells

Cell culture was performed at 37°C in 95% air/5% CO₂. THP-1 cells (European Collection of Cell Cultures, Salisbury, UK) were grown in RPMI-1640 containing 10% fetal calf serum, 2 mM glutamine, 10 $\mu\text{g}/\text{ml}$ streptomycin and 10 U/ml penicillin-G. MonoMac-6 cells (Inerlab Cell Line Collection, Genova, Italy) were grown in RPMI-1640 containing 10% fetal calf serum, 1% nonessential amino acids, 9 $\mu\text{g}/\text{ml}$ bovine insulin, 1 mM sodium pyruvate, 10 $\mu\text{g}/\text{ml}$ streptomycin and 10 U/ml penicillin-G.

Prior to the fluorimetric assay, cells were resuspended in fresh assay medium: CO₂-independent medium containing 1% bovine serum albumin. Cell viability was determined using trypan blue, with viability rates of >98% routinely obtained. Cells ($2 \times 10^5/\text{well}$) were dispensed into black NUNC polystyrene 96-well microtiter plates (VWR) with 5 μM of the FAM-TAMRA TNF peptide, in total volume of 80 μl . For inhibitor studies, test compounds dissolved in dimethylsulfoxide were dispensed to wells prior to the addition of substrate and cells. Final dimethylsulfoxide concentration was adjusted to 0.75% (v/v) in all wells. Hydroxamate-based metalloprotease inhibitors, BB94 (British Biotech Pharmaceuticals Ltd, Abingdon, UK), GM6001, GW280264X and GI254023X (Glaxo-SmithKline); and broad-spectrum cysteine (E-64), serine (Leupeptin) and aspartyl (H-KTEEISEVN-stat-VAEF-NH₂) protease inhibitors were tested. Peptide cleavage in the wells was monitored continuously for 30–60 min at 35°C using a Bio-Tek FLx800 microplate fluorescence reader (Bio-Tek Instruments, Vermont, USA) at λ_{ex} 485 nm and λ_{em} 535 nm, and analyzed by KC4 software (Erithacus Software Ltd).

Inhibition of proTNF Cleavage from THP-1 Cells

THP-1 cells were pretreated overnight with 100 ng/ml of interferon-gamma (R&D Systems) and then stimulated with 1 $\mu\text{g}/\text{ml}$ of lipopolysaccharide (LPS) for 3 h in the presence of titrated doses of GM6001 (0.05–128.5 μM). Soluble TNF levels in the

supernatants were determined by enzyme-linked immuno-sorbent assay using paired anti-TNF monoclonal antibodies (BD Biosciences, Oxford, UK).

Analysis of FAM-TAMRA Peptide Cleavage by Activated Cells

Interferon-gamma-primed THP-1 cells were stimulated with 1 $\mu\text{g}/\text{ml}$ of LPS for 3 h, and then analyzed using the FAM-TAMRA TNF peptide as described above. MonoMac-6 cells were directly stimulated with 30 ng/ml of phorbol myristate acetate (PMA) within the 96-well assay plates. PMA was added to the cell/substrate mixture immediately prior to the start of the assay, and real-time changes in fluorescent signal induced by PMA were monitored.

Flow Cytometry Analysis of Cell Surface TACE Expression

Cells were stained in the dark at 4°C for 30 min with phycoerythrin-conjugated anti-TACE ectodomain (clone 111633) or isotype-matched control antibodies (R&D Systems). Cells were fixed with 1% formaldehyde and analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, Oxford, UK).

Results

FAM-TAMRA Peptide Cleavage by Recombinant TACE and ADAM-10

We first tested the validity of the FAM-TAMRA TNF peptide *in vitro* using recombinant enzymes. Peptide cleavage was measured by real-time monitoring of fluorescent signal using a spectrofluorimeter. Hydrolysis of 5 μM FAM-TAMRA TNF peptide by 7 nM rTACE produced a four-fold increase above the background fluorescent signal over 60 min (Figure 1a). The reaction curve was linear over time with the rate of peptide cleavage effectively constant. No peptide cleavage was observed in the absence of enzyme, and complete inhibition of the cleavage was achieved by >6 μM of GM6001 with an IC₅₀ of 20.6 ± 1.2 (s.e.) nM ($n=4$). A $k_{\text{cat}}/K_{\text{m}}$ of $0.99 \pm 0.03 \mu\text{M}^{-1} \text{s}^{-1}$ was determined ($n=8$), which is >7-fold higher than the previously published $k_{\text{cat}}/K_{\text{m}}$ value of TACE for an Mca-SPLAQAVRSSSRK-(Dnp)-NH₂ peptide ($0.13 \pm 0.01 \mu\text{M}^{-1} \text{s}^{-1}$).¹² This implies that TACE can cleave the FAM-TAMRA TNF peptide more efficiently than the Mca-Dnp peptide of the same amino-acid sequence. The peptide could not be dissolved in the buffer solution at concentrations high enough to allow accurate determination of K_{m} and V_{max} .

ADAM-10, another protease of the ADAM family, has been reported to cleave TNF-based peptides.⁶ Hydrolysis of 5 μM FAM-TAMRA TNF peptide by 17 nM rADAM-10 over 60 min produced a 2.3-fold

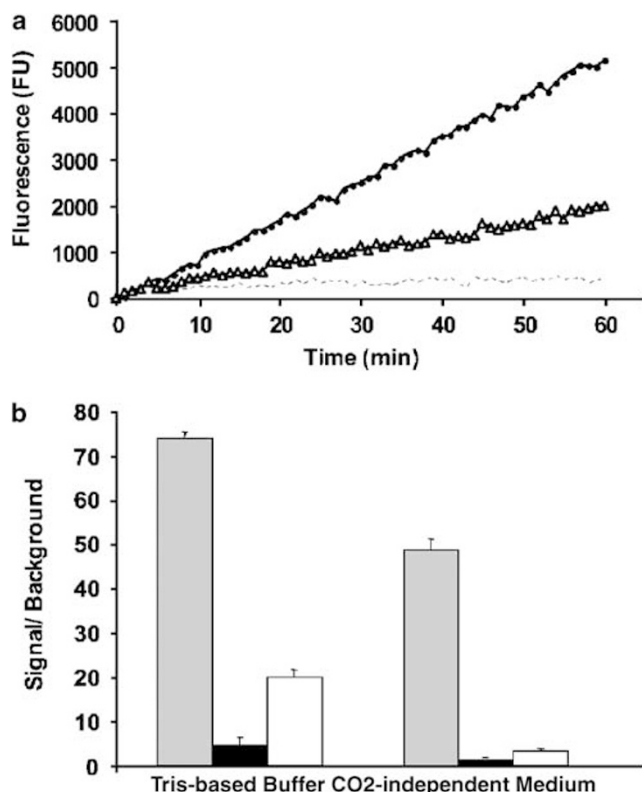


Figure 1 Validation of the FAM-TAMRA TNF peptide-based assay using recombinant enzymes. **(a)** Typical reaction curves of the peptide cleavage by rTACE (●) (7 nM) and rADAM-10 (△) (17 nM). The dashed line represents peptide cleavage in assay medium without any enzyme. Fluorescent unit (FU) was plotted against time with background fluorescent values at $t=0$ subtracted. **(b)** Signal intensity of FAM-TAMRA TNF peptide and cell media interference. Ratios of fluorescent signal between cleaved and uncleaved substrate by rTACE, are shown for FAM-TAMRA (gray), Dabcyl-Edans (black) and Mca-Dpa (white) peptides ($n=4$). Signal/background ratio for the FAM-TAMRA peptide was considerably higher than those for the other two peptides in both the tris-based assay buffer and CO₂-independent medium.

increase of the fluorescent signal above the background (Figure 1a). However, k_{cat}/K_m was $0.12 \pm 0.004 \mu\text{M}^{-1} \text{s}^{-1}$ for rADAM-10 ($n=8$), that is, >8 -fold lower than for TACE, indicating that ADAM-10 is less efficient at cleaving this peptide.

Signal Intensity of FAM-TAMRA Peptide and Cell Media Interference

We then investigated the signal intensity of FAM-TAMRA TNF peptide in comparison with Dabcyl-Edans and Mca-Dpa peptides, in assay solutions used for our *in vitro* as well as cell-based assays. Complete hydrolysis of these TNF peptides was achieved by overnight incubation with an excess dose of rTACE (50 nM for 10 μM substrate), and the ratio of fluorescent signal between the cleaved and uncleaved peptide, both diluted to 400 nM in each assay solution, was compared among the peptides.

In the tris-based assay buffer, peptide cleavage resulted in much larger increase in fluorescent signal with FAM-TAMRA (~ 74 -fold) than Dabcyl-Edans (~ 5 -fold) or Mca-Dpa (~ 20 -fold) peptides (Figure 1b). In CO₂-independent medium, signal enhancement due to peptide hydrolysis remained high with FAM-TAMRA peptide (~ 50 -fold), but was reduced to very low levels with both Dabcyl-Edans and Mca-Dpa peptides (<5 -fold). These results demonstrated that the FAM-TAMRA peptide provides superior signal/background ratio to Dabcyl-Edans or Mca-Dpa peptides, and that signal interference by cell culture media substantially hampers the sensitivity of Dabcyl-Edans or Mca-Dpa peptides, but not that of FAM-TAMRA peptide.

FAM-TAMRA Peptide Cleavage by Intact THP-1 Cells

We evaluated the cleavage of this FAM-TAMRA TNF peptide by THP-1 cells in viable whole-cell preparations. Cells were incubated at a density of 2×10^5 /well with 5 μM of FAM-TAMRA TNF peptide, and peptide cleavage was monitored at minute intervals for 30–60 min. The reaction curve was remarkably linear over time (Figure 2a), suggesting that the assay conditions are adequate for cell stability and constant substrate hydrolysis, as observed with rTACE. After an initial equilibration period, the rate of fluorescent signal increase was calculated over 10 min, as an estimation of enzyme activity (FU/min). Peptide cleavage in medium-only wells was negligible, and incubation of cells with a control nonspecific FAM-TAMRA peptide (FAM-SEVNLDAEFK-TAMRA) lacking the cleavage site of human proTNF by TACE (Ala-Val bond),⁸ did not produce any signal, demonstrating that the observed fluorescence increase is peptide sequence-specific.

To assess the quantitative nature of this assay, different densities of THP-1 cells (from 1×10^4 to 2×10^6 /well) were incubated with 5 μM of the FAM-TAMRA TNF peptide, and enzyme activity calculated from each reaction curve. Increasing cell density resulted in a proportional increase in the enzyme activity (Figure 2b), indicating a linear relationship between the amount of cellular enzyme in the reaction mixture and the degree of peptide hydrolysis. The assay was highly sensitive, with enough signal detected for reliable calculation of the enzyme activity at cell densities as low as 1×10^5 /well. The assay was reproducible with intra-assay variability of $<5\%$ and interassay variability of $<20\%$.

Effect of Inhibitors on FAM-TAMRA Peptide Cleavage

To characterize the enzymatic profile of FAM-TAMRA TNF peptide cleavage by THP-1 cells, we investigated the effects of addition of various protease inhibitors to the cell/peptide mixture. Saturating concentrations of broad-spectrum metal-

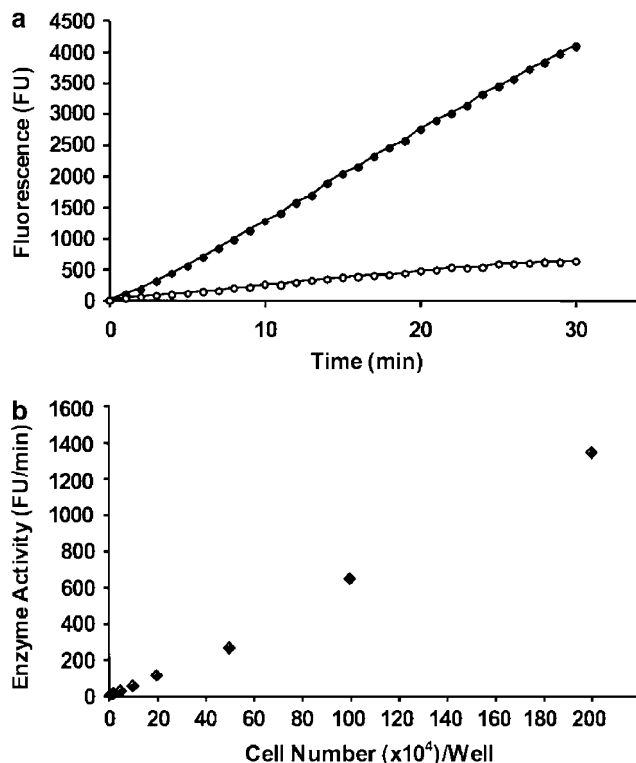


Figure 2 Validation of the FAM-TAMRA TNF peptide-based assay using live cells. (a) A typical reaction curve of the peptide cleavage by live THP-1 cells (●) and the effect of the metalloprotease inhibitor BB94 (○). In this case, BB94 (100 μ M) showed 83% reduction of the rate of fluorescent signal increase. (b) Relationship between the enzyme activity and the number of THP-1 cells in the reaction mixture. With a fixed concentration of the peptide, increasing cell density resulted in a proportional increase in the enzyme activity (calculated from each reaction curve).

loprotease inhibitors BB94 and GM6001 resulted in marked reduction of the rate of fluorescent signal increase (Figure 2a). The inhibition was $79 \pm 2\%$ ($n = 4$) with 100 μ M BB94 and $81 \pm 4\%$ ($n = 3$) with 128.5 μ M GM6001. In contrast, inhibitors of cysteine (E-64), serine (Leupeptin) and aspartyl (H-KTEEI-SEVN-stat-VAEF-NH₂) proteases did not produce any changes in peptide hydrolysis (data not shown).

We then determined the dose-response curve for GM6001-mediated inhibition of the peptide cleavage, and compared it with the dose-response curve for GM6001-mediated inhibition of proTNF cleavage, that is, inhibition of soluble TNF release from LPS-stimulated cells into culture supernatants (Figure 3a). The inhibition profiles of the synthetic and native substrate cleavage by THP-1 cells were very similar, with an IC_{50} of $5.5 \pm 3 \mu$ M for the FAM-TAMRA TNF peptide cleavage and $13 \pm 15 \mu$ M for the proTNF cleavage ($n = 3$). We further studied the effects of two inhibitors of more specific spectra, that is, the dual TACE/ADAM-10 inhibitor GW280264X, and the ADAM-10 selective inhibitor GI254023X, both previously used to determine the roles of TACE and ADAM-10 in chemokine cleavage.^{18,19} Dose-response curves demonstrated that

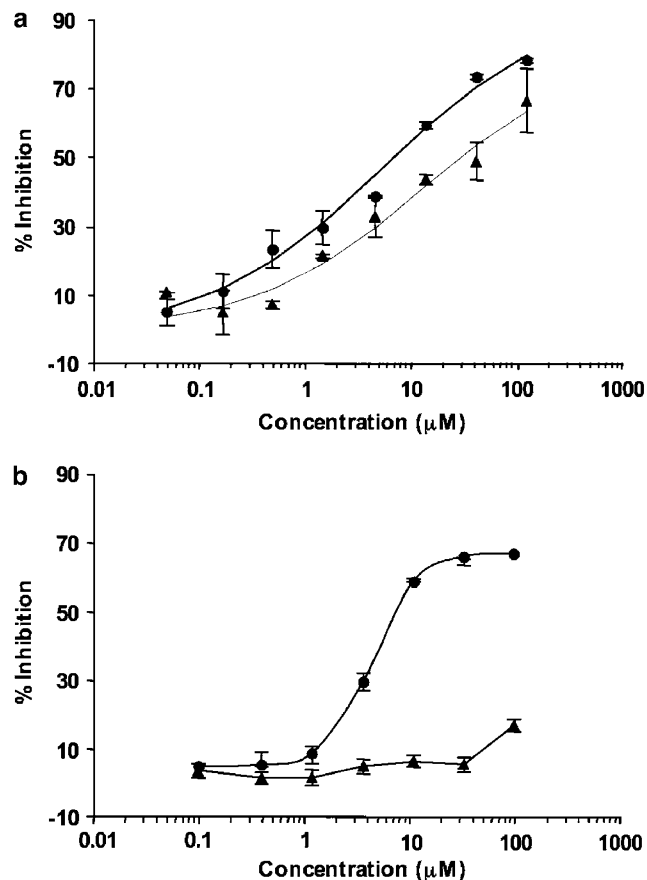


Figure 3 Validation of the FAM-TAMRA TNF peptide-based assay for specific detection of TACE enzyme activity. (a) GM6001-mediated inhibition of the peptide cleavage by THP-1 cells (●) was compared to GM6001-mediated inhibition of proTNF cleavage from the surface of THP-1 cells (▲). Both curves were similar with an IC_{50} of $5.5 \pm 3 \mu$ M for the peptide cleavage and $13 \pm 15 \mu$ M for the proTNF cleavage ($n = 3$). (b) GW280264X (●), a dual TACE/ADAM-10 inhibitor, induced substantial inhibition in the peptide cleavage at concentrations $> 10 \mu$ M (IC_{50} $4.5 \pm 0.3 \mu$ M), while selective ADAM-10 inhibitor GI254023X (▲) induced only minimal inhibition ($IC_{50} > 100 \mu$ M) ($n = 6$). Data presented as mean \pm s.e.

the peptide cleavage by THP-1 cells was efficiently inhibited by the dual inhibitor but not by the ADAM-10 inhibitor (Figure 3b). GW280264X showed considerable inhibition (60–68%) at concentrations $> 10 \mu$ M with an IC_{50} of $4.5 \pm 0.3 \mu$ M, while same concentrations of GI254023X induced only 5–15% inhibition with an $IC_{50} > 100 \mu$ M ($n = 6$). These pharmacological profiles indicated that TACE is most likely the enzyme responsible for the majority of the FAM-TAMRA TNF peptide cleavage by intact THP-1 cells.

FAM-TAMRA Peptide Cleavage by Activated THP-1 and MonoMac-6 Cells

To investigate whether the FAM-TAMRA TNF peptide is useful not only for detection of basal

TACE activity on unstimulated cells, but also for detection of 'changes' in cellular TACE activity upon cell stimulation, we studied the peptide cleavage by activated THP-1 cells. Cells pretreated with interferon-gamma and stimulated with LPS for 3 h produced a substantial (3.3-fold) increase in the rate of peptide hydrolysis, compared with nonstimulated (interferon-gamma only) cells (Figure 4a). Addition of 33 μ M GW280264X to LPS-stimulated

THP-1 cells resulted in $79 \pm 0.5\%$ reduction in peptide cleavage while GI254023X at the same concentration produced only $5 \pm 4\%$ inhibition ($n=5$). This inhibition profile, essentially similar to unstimulated THP-1 cells, implies that the observed LPS-induced increase in peptide cleavage is also largely attributable to increased TACE activity. Interestingly, flow cytometric analysis showed that cell surface TACE expression did not increase upon LPS stimulation (Figure 4b), suggesting that increased TACE activity on THP-1 cells could occur independently of the enzyme surface expression.

MonoMac-6 cells secrete little soluble TNF when stimulated with LPS alone, but produce an immediate (within 20–30 min) large release of soluble TNF when pretreated with LPS and then stimulated with PMA,²⁰ presumably due to rapid upregulation of proTNF shedding. To assess whether such PMA-induced rapid changes in TACE activity could be demonstrated using the FAM–TAMRA TNF peptide, MonoMac-6 cells were assayed with the peptide in the presence of PMA (30 ng/ml) and changes in rate of peptide hydrolysis were monitored in comparison with unstimulated cells. We observed a rapid increase in the rate of peptide hydrolysis 5 min after the addition of PMA, becoming distinct by 10–15 min (Figure 3c). This provides evidence that the assay is highly sensitive and capable of detecting dynamic real-time changes in cellular TACE activity upon cell activation.

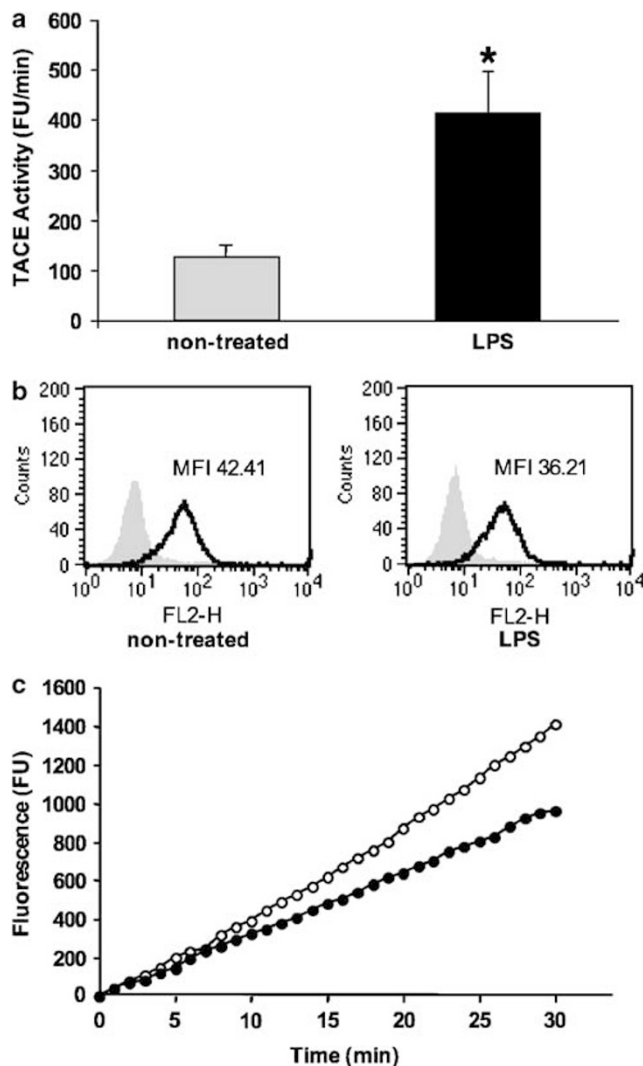


Figure 4 Detection of TACE enzyme activity in activated cells. (a) THP-1 cells that were treated with LPS 1 μ g/ml for 3 h showed considerable increase in the rate of FAM–TAMRA peptide cleavage compared to nontreated controls ($n=3$, $*P<0.05$, t -test). Data presented as mean \pm s.e. (b) Flow cytometric analysis of LPS-treated cells demonstrated that cell surface TACE expression did not increase, but rather showed a tendency to decrease, compared to nontreated cells. The values of mean fluorescence intensity (MFI) of anti-TACE antibody staining are shown, with the isotype control (gray fill) MFI values subtracted. (c) MonoMac-6 cells were assayed with the peptide in the presence of PMA 30 ng/ml (\circ), and real-time changes in peptide cleavage were monitored. A higher rate of hydrolysis of the FAM–TAMRA peptide in PMA-treated cells was observed in comparison with nontreated cells (\bullet), and this was already evident after 5–10 min (single representative experiment, $n=3$).

FAM–TAMRA Peptide Cleavage and TACE Expression in MonoMac-6 Cells

Using flow cytometry, we have observed two distinct subpopulations of MonoMac-6 cells, high side scatter cells with significant surface TACE expression, and low side scatter cells expressing negligible amounts of TACE (<3 mean fluorescence intensity). With continuous culture, the percentage of high side scatter cells, as well as the amount of TACE expressed by the cells, decreased to low or undetectable levels. Application of the FAM–TAMRA peptide assay to these MonoMac-6 cells at different passages exhibited a correlation (R^2 0.95, $P<0.01$, $n=7$) between cell surface TACE expression and rates of peptide cleavage (Figure 5).

Discussion

Analysis of the biological role of TACE requires measurement of its enzyme activity *in situ*, that is, on the cell surface. However, previous methodologies have failed to provide reliable direct continuous measurement of TACE activity in intact cells. Using a newly designed TNF-based FRET peptide with the fluorophore pair FAM–TAMRA, we have developed an assay that enabled for the first time accurate and

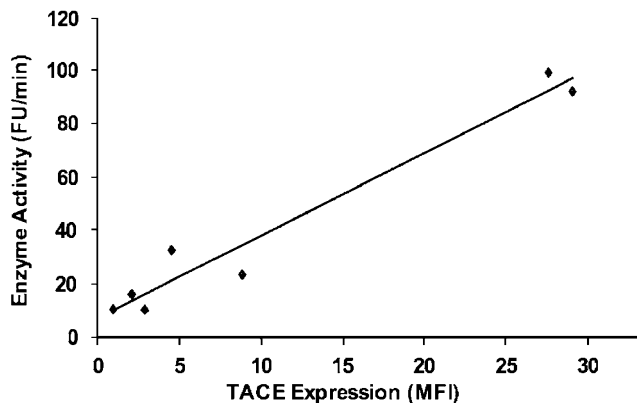


Figure 5 Linear regression analysis of the relationship between surface TACE expression on MonoMac-6 cells and FAM-TAMRA peptide cleavage. Different passages of the cells were analyzed for surface TACE expression using flow cytometry, expressed by mean of fluorescent intensity (MFI) of the total cell population with values of isotype control subtracted, and for enzyme activity using FAM-TAMRA peptide. R^2 was 0.95 ($n = 7$, $P < 0.01$).

reproducible determination of continuous real-time TACE activity on the surface of intact viable cells.

Up to now, successful measurement of TACE activity in intact cells has only been achieved using HPLC,¹⁶ which precludes continuous measurement of the enzyme's activity. Although the use of TNF-based FRET peptides for real-time measurement of recombinant and isolated native TACE activity is well established, their application to cell lysates or whole-cell preparations has been unsuccessful, mainly due to low assay sensitivity and specificity.¹⁵ By synthesizing a new TNF-based FRET peptide with the fluorophore pair FAM-TAMRA, we have overcome the problems of the previously used FRET peptides. The unique physical properties of FAM, that is, greater fluorescent intensity and lower signal interference, allow detection of the signal in cell suspensions with much better sensitivity, as reflected by a considerably higher signal/background ratio of FAM-TAMRA peptide than that of Dabcyl-Edans or Mca-Dpa-based peptides. Furthermore, the signal/background ratio remained high even in cell culture media despite some signal interference, that is, >10-fold higher than that for the Dabcyl and Mca peptides, which makes FAM-TAMRA peptide more suitable for measurement of cellular TACE activity.

Since our assay is based on the substrate specificity of an enzyme, but not on its immunological properties, there is always some concern whether the peptide substrate used may have some cross-reactivity with other enzymes. In particular, although TACE is considered to be the major physiological TNF sheddase, responsible for 90% of its cleavage *in vivo*,³ other cell surface sheddases in the metalloprotease family, such as ADAM-10 and matrix metalloprotease (MMP)-7, have been shown to possess some, albeit small, TNF-cleaving activity.^{6,21,22} However, we found that specificity constant (k_{cat}/K_m) for the cleavage of FAM-TAMRA

TNF peptide by rADAM-10 was eight-fold lower than that by rTACE, consistent with previous published data by Moss *et al*.⁶ Mohan *et al*.²² reported that the k_{cat}/K_m for the cleavage of a similar peptide with a TACE-sensitive TNF sequence was 200-fold lower with rMMP-7 than rTACE. These *in vitro* experiments using recombinant enzymes suggest that if hydrolysis of the FAM-TAMRA peptide in cell-based assays is metalloprotease-dependent, then the observed enzyme activity should be attributable mainly to TACE, rather than ADAM-10 or MMP-7.

In order to directly evaluate TACE specificity of our cellular assay, we first studied how FAM-TAMRA TNF peptide cleavage was affected by the broad-spectrum metalloprotease inhibitors BB94 and GM6001, which have been shown to efficiently inhibit proTNF shedding from cells *in vivo*.^{23,24} Approximately 80% inhibition was observed with these compounds while other protease inhibitors were completely ineffective, indicating that the peptide cleavage was almost entirely metalloprotease-dependent. We also found that the dose-response profile of GM6001 inhibition of the FAM-TAMRA TNF peptide cleavage was remarkably similar to that of the *in vivo* proTNF cleavage, suggesting that TACE, which is responsible for the cleavage of proTNF from cell surface, also cleaves the synthetic TNF peptide. Finally, to exclude a potential role of ADAM-10 in the peptide cleavage, we compared the inhibitory effects of two new ADAM inhibitors with different specificity. The dual TACE/ADAM-10 inhibitor GW280264X substantially reduced the peptide cleavage, while the ADAM-10 selective inhibitor GI254023X produced minimal inhibition. Thus, despite the potential crossreactivity of the FAM-TAMRA TNF peptide with other sheddases on the cell surface, the results strongly suggest that the observed proteolytic activity in our cellular assay could be largely ascribed to TACE. The correlation between the peptide cleavage and the levels of surface TACE expression found in MonoMac cells is consistent with this hypothesis. Clearly, some validation experiments may be necessary if applying this assay to other cell types in which the relative levels of active TACE or other cell surface metalloproteases are different.

We further investigated the suitability of this assay to detect 'changes' in TACE activity in viable intact cells. In the present study, a substantial increase in FAM-TAMRA TNF peptide cleavage was detected in THP-1 cells upon LPS stimulation. This effect of LPS was not associated with significant changes in surface TACE expression, suggesting that TACE catalytic activity *per se* (TACE activity per enzyme molecule) was upregulated by LPS. Although it is possible that upregulation of other TNF sheddases expression, in particular ADAM-10, were responsible for the increased activity, addition of the dual TACE/ADAM-10 as well as ADAM-10 selective inhibitors to the LPS-treated cells indi-

cated that the LPS-induced increase in enzyme activity was largely ascribed to enhanced activity of TACE, rather than ADAM-10 or other nonmetalloprotease enzymes. In addition, dynamic very rapid changes in enzyme activity were observed in MonoMac-6 cells upon PMA stimulation, consistent with the previously proposed concept of rapid induction of TNF shedding activity by PMA in these cells.²⁰ The inferior signal/background ratio of traditional FRET-based TNF peptides, as compared to the FAM-TAMRA peptide, would have precluded their use in studying such a phenomenon. Thus, our cellular assay provides quantitative, real-time data of the catalytic performance of TACE, both at basal and induced states. The ability to perform real-time measurements would be of particular benefit for investigating the regulation of TACE under acute inflammatory conditions.

TACE-mediated shedding occurs constitutively at low rates, and can be rapidly upregulated with stimuli such as phorbol esters or MAP kinase cascade activators.^{25–27} Like other ADAMs, TACE requires cleavage of its prodomain to become a 'mature' fully active protease.^{6–8} It has been suggested that constitutive removal of the prodomain occurs intracellularly and only processed, active TACE is expressed on the cell surface, as is the case for THP-1 cells.^{28,29} Several mechanisms have been postulated to explain how TACE activity is modulated, for example, phosphorylation of the intracellular domain and association with cytoplasmic proteins^{7–9} that could explain how proteolytic activity of 'mature' TACE could be upregulated following cell activation irrespective of changes in cell surface expression. In contrast, others reported that prodomain removal is not a prerequisite for surface TACE expression³⁰ and that reactive oxygen species may mediate prodomain removal and activation on the cell surface.^{26,27} Thus, regulation of TACE activity appears to be a complex and dynamic process, involving various biochemical as well as signaling pathways. Development of a reliable direct methodology for measurement of TACE catalytic activity in live cells is essential, and our new FRET peptide assay represents a step forward to better understanding of the mechanisms of TACE regulation *in vivo*.

In summary, this study reports the development and validation of a new TACE activity assay which, to our knowledge, for the first time enables accurate, quantitative and reproducible determination of continuous real-time TACE activity on the surface of live cells, both at resting and activated states. The assay offers a valuable tool for the *in vivo* analysis of TACE activity, potentially leading to new insights into the biological roles of TACE and TNF in the pathophysiology of inflammatory diseases. Moreover, the principles developed in this study, that is, FAM-TAMRA FRET technology and assessment by pharmacological/enzymatic profiling, could be further extrapolated to any surface enzymes with

defined substrates, facilitating the establishment of new assays to measure their activity on the surface of live cells.

Acknowledgements

We thank Professor G Murphy and Dr K Moore for their invaluable advice throughout this work, and Dr S Ratcliffe for peptide synthesis. This work was partly supported by grants from Meningitis Research Foundation (#07/00), Wellcome Trust (#057459), Chelsea and Westminster Healthcare NHS Trust Charity, Westminster Medical School Research Trust, and GlaxoSmithKline.

References

- 1 Werb Z, Yan Y. A cellular striptease act. *Science* 1998;282:1279–1280.
- 2 Mullberg J, Althoff K, Jostock T, *et al*. The importance of shedding of membrane proteins for cytokine biology. *Eur Cytokine Netw* 2000;11:27–38.
- 3 Black RA, Rauch CT, Kozlosky CJ, *et al*. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature* 1997;385:729–733.
- 4 Moss ML, Jin SL, Milla ME, *et al*. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- α . *Nature* 1997;385:733–736.
- 5 Peschon JJ, Slack JL, Reddy P, *et al*. An essential role for ectodomain shedding in mammalian development. *Science* 1998;282:1281–1284.
- 6 Moss ML, White JM, Lambert MH, *et al*. TACE and other ADAM proteases as targets for drug discovery. *Drug Discov Today* 2001;6:417–426.
- 7 Black RA. Tumor necrosis factor- α converting enzyme. *Int J Biochem Cell Biol* 2002;34:1–5.
- 8 Mezyk R, Bzowska M, Bereta J. Structure and functions of tumor necrosis factor- α converting enzyme. *Acta Biochim Pol* 2003;50:625–645.
- 9 Blobel CP. ADAMs: key components in EGFR signaling and development. *Nat Rev Mol Cell Biol* 2005;6:32–43.
- 10 Stack MS, Gray RD. Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. *J Biol Chem* 1989;264:4277–4281.
- 11 Knight CG, Willenbrock F, Murphy G. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett* 1992;296:263–266.
- 12 Amour A, Slocumbe PM, Webster A, *et al*. TNF- α converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* 1998;435:39–44.
- 13 Jin G, Huang X, Black R, *et al*. A continuous fluorimetric assay for tumor necrosis factor- α converting enzyme. *Anal Biochem* 2002;302:269–275.
- 14 Neumann U, Kubota H, Frei K, *et al*. Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. *Anal Biochem* 2004;328:166–173.
- 15 Becker BF, Gilles S, Sommerhoff CP, *et al*. Application of peptides containing the cleavage sequence of pro-

- TNF α in assessing TACE activity of whole cells. *Biol Chem* 2002;383:1821–1826.
- 16 Doedens JR, Mahimkar RM, Black RA. TACE/ADAM-17 enzymatic activity is increased in response to cellular stimulation. *Biochem Biophys Res Commun* 2003;308:331–338.
- 17 Pope AJ, Haupts UM, Moore KJ. Homogeneous fluorescence readouts for miniaturized high-throughput screening: theory and practice. *Drug Discov Today* 1999;4:350–362.
- 18 Abel S, Hundhausen C, Mentlein R, *et al*. The transmembrane CXC-chemokine ligand 16 is induced by IFN- γ and TNF- α and shed by the activity of the disintegrin-like metalloproteinase ADAM10. *J Immunol* 2004;172:6362–6372.
- 19 Hundhausen C, Misztela D, Berkhout TA, *et al*. The disintegrin-like metalloproteinase ADAM10 is involved in constitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell–cell adhesion. *Blood* 2003;102:1186–1195.
- 20 Pradines-Figueras A, Raetz CR. Processing and secretion of tumor necrosis factor alpha in endotoxin-treated Mono Mac 6 cells are dependent on phorbol myristate acetate. *J Biol Chem* 1992;267:23261–23268.
- 21 Haro H, Crawford HC, Fingleton B, *et al*. Matrix metalloproteinase-7-dependent release of tumor necrosis factor- α in a model of herniated disc resorption. *J Clin Invest* 2000;105:143–150.
- 22 Mohan MJ, Seaton T, Mitchell J, *et al*. The tumor necrosis factor- α converting enzyme (TACE): a unique metalloproteinase with highly defined substrate selectivity. *Biochemistry (Moscow)* 2002;41:9462–9469.
- 23 Solorzano CC, Ksontini R, Pruitt JH, *et al*. Involvement of 26-kDa cell-associated TNF- α in experimental hepatitis and exacerbation of liver injury with a matrix metalloproteinase inhibitor. *J Immunol* 1997;158:414–419.
- 24 Santucci MB, Ciaramella A, Mattei M, *et al*. Batimastat reduces *Mycobacterium tuberculosis*-induced apoptosis in macrophages. *Int Immunopharmacol* 2003;3:1657–1665.
- 25 Fan H, Derynck R. Ectodomain shedding of TGF- α and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades. *EMBO J* 1999;18:6962–6972.
- 26 Zhang Z, Kolls JK, Oliver P, *et al*. Activation of tumor necrosis factor- α -converting enzyme-mediated ectodomain shedding by nitric oxide. *J Biol Chem* 2000;275:15839–15844.
- 27 Zhang Z, Oliver P, Lancaster JJ, *et al*. Reactive oxygen species mediate tumor necrosis factor alpha-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate. *FASEB J* 2001;15:303–305.
- 28 Schlondorff J, Becherer JD, Blobel CP. Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem J* 2000;347(Part 1):131–138.
- 29 Doedens JR, Black RA. Stimulation-induced down-regulation of tumor necrosis factor- α converting enzyme. *J Biol Chem* 2000;275:14598–14607.
- 30 Peiretti F, Canault M, Deprez-Beauclair P, *et al*. Intracellular maturation and transport of tumor necrosis factor alpha converting enzyme. *Exp Cell Res* 2003;285:278–285.