INFLAMMATORY BOWEL DISEASE

Tumour necrosis factor α converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease

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Background: Anti-tumour necrosis factor α (TNF- α) antibodies are effective in Crohn's disease and perhaps ulcerative colitis but antigenicity and the high cost have raised interest in other strategies to block TNF- α . These include the TNF- α converting enzyme (TACE) which releases soluble TNF- α from transmembrane pro-TNF- α .

Aim: To investigate whether TACE activity is present in human colonic mucosa.

Materials and methods: Detergent extracts of cell membranes from colonic biopsies were obtained from 12 controls and 28 patients with inflammatory bowel disease. Enzyme activity was measured by hydrolysis assays using pro-TNF- α or oligopeptide substrates spanning the known pro-TNF- α cleavage site at Ala(76)-Val(77). Cleavage products were identified by western blotting, high pressure liquid chromatography, or mass spectrometry. TACE protein was localised by immunohistochemistry and identified by western blotting of detergent extracts from purified lamina propria mononuclear cells (LPMNC) or epithelial cells.

Results: Detergent extracts released TNF- α from pro-TNF- α and cleaved a model oligopeptide as predicted. Substrate hydrolysis was sensitive to known TACE/matrix metalloproteinase (MMP) inhibitors, but not trocade which has low activity against TACE. The median TACE level was increased in active ulcerative colitis (147 arbitrary units (AU)/mg; $p < 0.01$) but not in Crohn's disease (81 AU/mg) compared with controls (79 AU/mg). Both the full length proform and the active form of TACE protein were expressed in LPMNC cells and epithelial cells.

Conclusions: Functional TACE activity is ubiquitously expressed in the human colon and increased in ulcerative colitis, raising interest in MMP inhibitors targeting TACE.

The therapeutic gain of anti-tumour necrosis factor α (TNF- α) antibody treatment in Crohn's disease,¹⁻⁷ and perhaps also in ulcerative colitis,⁸ indicates that increased levels of TNF- α play a central pathogenic role in inflammatory bowel disease (IBD). Anti-TNF- α antibody treatment is well tolerated but has a number of shortcomings in clinical practice. These include antigenicity of the antibody components, rare occurrence of a lupus-like syndrome, requirement for parenteral administration, and high cost.⁹ These issues have raised interest in the development of alternative therapeutic strategies to block TNF- α activity.

TNF- α is translated as a 26 kDa type II transmembrane precursor protein which requires specific proteolytic cleavage in the extracellular domain at the Ala76-Val77 bond to release the soluble and presumably biologically active 17 kDa N terminal part of pro-TNF- α . The protease responsible for this cleavage has recently been identified as a membrane anchored multidomain metalloproteinase called TNF- α converting enzyme (TACE).¹⁰⁻¹¹ TACE (ADAM 17) belongs to the ADAM (*a* disintegrin and metalloproteinase) family of cell surface proteins which are involved in diverse functions such as fertilisation, myogenesis, neurogenesis, neutrophil migration, and ectodomain shedding of cell surface proteins like TNF- α .¹²⁻¹⁴ Northern blot analysis has shown strong expression of mRNA for TACE in several organs such as the heart, placenta, testes, ovaries, and small bowel, whereas weaker expression was observed in libraries of a variety of other human organs, including the colon.¹⁰

Using the more sensitive reverse transcription-polymerase chain reaction technique, we found that TACE mRNA was ubiquitously expressed in human colonic mucosa and that transcript levels were upregulated in IBD.¹⁵ Preliminary data

also suggested the presence of TACE activity¹⁵; however, other matrix metalloproteinases (MMP) present in colonic mucosa¹⁶⁻¹⁸ can also cleave pro-TNF- α in vitro,¹⁹ and the enzyme responsible for TNF- α release in the colon has not yet been identified. Here, we provide evidence that TACE activity is present in human colonic mucosa and increased in ulcerative colitis.

METHODS

Materials

Oligopeptides with the sequence ac-SPLAQAVRSSSR-NH₂ or dinitrophenol (dnp)-SPLAQAVRSSSRTPS-NH₂ corresponding to the known pro-TNF- α cleavage site by TACE at Ala (76)-Val (77) were synthesised by commercial solid phase synthesis (KE Jørgensen, Copenhagen, Denmark) and purified in our laboratory using reverse phase high pressure liquid chromatography (HPLC) on a preparative nucleosil 5 μ C18 column (Pharmacia Biotek, Denmark). Purity was at least 95%, as

Abbreviations: ADAM, a disintegrin and metalloproteinase; AU, arbitrary units; BSA, bovine serum albumin; DMSO, dimethylsulphoxide; dnp, dinitrophenol; EDTA, ethylene-diamine-tetraacetic acid; EGTA, ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; GST, glutathione-S-transferase; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; IBD, inflammatory bowel disease; LPMNC, lamina propria mononuclear cells; MMP, matrix metalloproteinase; MT, membrane type; PBS, phosphate buffered saline; PE, phycoerythrin; SDS, sodium dodecyl sulphate; TACE, TNF- α converting enzyme; TLCK, N α -p-tosyl-L-lysine chloromethyl ketone; TNF- α , tumour necrosis factor α .

judged by HPLC analysis. Peptides for identification of breakdown products in HPLC analysis were prepared in the same way. Recombinant human TACE, recombinant glutathione-S-transferase (GST) pro-TNF- α substrate, and the MMP inhibitors CH4474, BB 94, and trocade, were obtained from Caltech Chiroscience (Cambridge, UK). A goat polyclonal antibody against a peptide mapping the carboxy terminus of human TACE (designated C-15 by the manufacturer) and an epitope specific blocking peptide were obtained from Santa Cruz Biotechnology (UK).

Patients

Colonoscopic biopsies were obtained from 18 patients with ulcerative colitis and 10 patients with Crohn's disease according to standardised diagnostic criteria.^{20, 21} Fifteen males and 13 females with a median age of 46 years (range 19–72) were included. Three patients were receiving oral prednisolone (12.5–30 mg/day) at the time of the study and only one was receiving azathioprine (150 mg/day). Six patients were receiving topical treatment with a prednisolone enema (25 mg/day) or a 5-aminosalicylic acid suppository. Twenty four patients were maintained on an oral 5-aminosalicylic acid containing drug (1–3 g/day). Disease activity was graded as described previously.^{20, 21} The control group consisted of three healthy controls and nine patients with no signs of neoplastic or inflammatory disease undergoing routine colonoscopy. Nine males and three females with a median age of 57 years (range 25–78) were studied. In patients with IBD, biopsies were collected from endoscopically inflamed or non-inflamed colonic mucosa, or both, using standard biopsy forceps (Olympus, Japan). Biopsies were immediately washed in isotonic saline and stored in 200 μ l of Tris buffer at -80°C for later detergent extraction of cell membrane enzyme activity. For western blotting and immunohistochemical studies, biopsies were collected from a further nine control patients, three males and six females, with a median age of 54 years (range 25–73). These biopsies were processed as described below. Permission for collection of biopsies was obtained from the regional ethics committee and all participants gave informed and written consent.

Detergent extraction of TACE activity from cell membranes

Five biopsies (average 5 mg each) were homogenised by hand in a glass to glass homogeniser (clearance 0.1 mm) in 250 μ l of Tris buffer (10 mM) at pH 7.5. Crude membranes were separated by precipitation at 15 000 g_{av} for 20 minutes after a 15 minute incubation period on ice after addition of CaCl_2 (10 mM) to a volume of 1 ml. The resulting pellet was dissolved in 50 μ l of 1% Nonidet P-40 (Sigma, St Louis, Missouri, USA) at $0-5^{\circ}\text{C}$ for one hour followed by centrifugation at 15 000 g_{av} for five minutes. The supernatant used for enzyme analysis is referred to in the following as detergent extract. For initial HPLC experiments, a pool of detergent extract was established from normal colonic tissue obtained in a patient undergoing surgical resection.

Pro-TNF- α hydrolysis assay

Recombinant human TACE or detergent extract (0.1 ng) was incubated for 60 minutes at 37°C with a recombinant GST pro-TNF- α substrate (6 ng) in a total incubation volume of 20 μ l. The incubation volume contained the following buffers: Tris (10 mM, pH 7.5), MgCl_2 (1 mM), CaCl_2 (0.2 mM), and protease inhibitors (pepstatin A (10 μM), leupeptin (10 μM), soybean trypsin inhibitor (1 $\mu\text{g}/\text{ml}$), trasylol (100 units/ml), and α -1-antitrypsin (0.2 mg/ml)) (all from Sigma). The inhibitory effects of ethylene-diamine-tetraacetic acid (EDTA) (5 mM), a zinc (and metal) chelating agent, and CH4474 (1 $\mu\text{g}/\text{ml}$), a MMP/TACE inhibitor, were studied in separate experiments.^{22, 23} The reaction was stopped by addition of sam-

ple loading electrophoresis buffer (5 μ l) with sodium dodecyl sulphate (SDS) (2.5%) followed by immediate heating to 95°C . Electrophoresis was performed by application of 4 μ l samples on a 20% continuous acrylamide gel using the Phast system (Pharmacia Biotech, Uppsala, Sweden). Western blotting was performed using electrophoretic transfer onto nitrocellulose membranes (Biorad, Sundbyberg, Sweden) followed by inactivation of the nitrocellulose membrane by incubation for one hour in 2% bovine serum albumin (BSA) (RIA grade; Sigma). Cleavage products of pro-TNF- α were detected by rabbit-anti-GST (AH Diagnostic, Aarhus, Denmark) and rabbit-antihuman TNF- α (Sigma), both diluted 1:4000 in BSA (1%). The secondary goat-antirabbit antibody (Dako, Copenhagen, Denmark) was also diluted 1:4000 in 1% BSA. Detection was performed with ECL western blotting reagents (Amersham Pharmacia Biotech, Denmark) following the manufacturer's instructions. Gels were photographed using a Polaroid camera designed for the purpose (Amersham, UK).

A modification of the assay was used in separate experiments to study the effect of $N\alpha$ -p-tosyl-L-lysine-chloromethyl ketone (TLCK), a serine protease inhibitor, on recombinant TACE activity. This assay was based on direct detection of substrate breakdown using silver staining to demonstrate substrate and products. The only other modification was an increase in the amount of GST-pro-TNF- α to 0.5 μg . Electrophoresis and silver staining were performed according to the manual for the Phast system.

Oligopeptide hydrolysis assays

Substrate peptide with either the sequence ac-SPLAQAVRSSSR-NH₂ or dnp-SPLAQAVRSSSRTPS-NH₂ (0.5 $\mu\text{g}/\text{ml}$) was added to the incubation medium to start the reaction and incubated at 37°C for 60 minutes. The incubation buffer and protease inhibitors were the same as described above. The total incubation volume was 20 μ l and the sample protein concentration ranged from 3 to 9 mg/ml. Freshly dissolved TLCK (10 $\mu\text{g}/\text{ml}$), the zinc chelating agents, phenantroline (5 mM), and EDTA (5 mM) (without MgCl_2 and CaCl_2), or one of the MMP inhibitors, CH4474 (1 $\mu\text{g}/\text{ml}$), BB94 (10 $\mu\text{g}/\text{ml}$), or trocade (5 μM), were added to the buffer in some experiments. CH4474 and BB94 are broad spectrum MMP inhibitors that also inhibit TACE. In contrast, trocade has only low activity against TACE but inhibits most MMPs.^{22, 23} CH4474 or BB94 was reconstituted in dimethylsulphoxide (DMSO) at a final concentration of 0.05%. The same DMSO concentration was used in parallel control experiments. The reaction was stopped by addition of 900 μ l of HCl (0.1 M). Peptide breakdown was determined by reverse phase HPLC using a 125 $\times$ 4 mm Machery-Nagel MN-C18 5 μ silica column (Machery Nagel, Germany). Separation was monitored at 215 nm for unlabelled peptide substrate, at 340 nm for dnp labelled peptide substrate, and expressed as arbitrary units (AU) corresponding to the peak area in the chromatogram. The position of the breakdown products was identified by synthetic standards with the predicted sequences for cleavage in the Ala-Val position. The breakdown products from the unlabelled peptide substrate were also identified by mass spectrometry (M-Scan, Oxford, UK). Fractions for this analysis were obtained by fraction collection from the HPLC analysis using peak detection and pooling of samples from 20 analyses. Unpaired and paired data were compared using non-parametric statistical methods.

Immunohistochemistry

Formalin fixed paraffin embedded tissue sections were dewaxed in xylene for 10 minutes and rehydrated in ethanol. After inhibition of endogenous peroxidase by a solution of hydrogen peroxide in ethanol (30 minutes), sections were heated at 60°C overnight in 10 mM Tris 0.25 mM EDTA, pH 9.0. Non-specific reactions with endogenous biotin were

blocked using a biotin blocking system (Dako), and other non-specific reactions were blocked using Tris buffered saline (0.05 M Tris/HCl, pH 7.6, 0.15 M NaCl) and casein 0.5%. The primary goat anti-TACE antibody (1:1000) was applied for 12–20 hours at 4°C. Biotinylated antigoat rabbit immunoglobulin was used as a secondary antibody. Finally, avidin DH and biotinylated horseradish peroxidase H reagents (Vector Laboratories, Burlingame, California, USA) were applied and visualised using diaminobenzidine as a chromogen. The reaction was enhanced using biotinylated thymidine followed by further avidin-peroxidase treatment. The nuclei were counterstained with haematoxylin. The specificity of the immunohistochemical analysis was confirmed by substituting the primary antibody with unspecific goat immunoglobulins (Dako) on parallel sections.

Isolation of epithelial cells and lamina propria mononuclear cells

Colonic epithelial cells were isolated from 5–7 biopsies obtained from endoscopically normal colonic mucosa using short term (10 minutes) EDTA/EGTA (ethylene glycol-bis (β -aminoethyl ether)- N,N,N',N' -tetraacetic acid) treatment, as described in detail recently.^{24, 25} This method has been shown to release crypts and crypt fragments with less than 5% contaminating cells. The isolated cells were washed with phosphate buffered saline (PBS) and stored until analysis at –80°C in a buffer containing Tris (10 mM, pH 7.5), $MgCl_2$ (1 mM), $CaCl_2$ (0.2 mM), and protease inhibitors (pepstatin A (10 μ M), leupeptin (10 μ M), soybean trypsin inhibitor (1 μ g/ml), trasylol (100 units/ml), and α -1-antitrypsin (0.2 mg/ml)). All reagents were obtained from Sigma.

Lamina propria mononuclear cells (LPMNC) were isolated from 7–10 biopsies, essentially as described previously.²⁶ Epithelial cells were removed using 60 minute EDTA/EGTA treatment followed by overnight incubation in the presence of 48 U/ml type I collagenase (Sigma) in RPMI 1640 medium (Gibco-BRL, UK). LPMNC were separated by passage through a cell strainer (100 μ nylon mesh; Falcon, UK), and purified by centrifugation on a Lymphoprep gradient (Nycomed, Norway). Cells were either suspended in 100 μ l PBS and applied to fluorescent flow cytometry or suspended in a 10 μ l protease inhibitor buffer (as above) and kept at –80°C for later western blot analysis. Flow cytometry analysis (FAC-Scan; Becton-Dickinson, Massachusetts, USA) of LPMNC was carried out using fluorescein isothiocyanate (FITC) conjugated or phycoerythrin (PE) conjugated antibodies against CD45 (PE) or CD68 (FITC) (Dako). In a representative experiment, >95% of 50 000 cells counted were CD45 positive. Further analysis showed a total of 42% lymphocytes estimated by forward scatter and side scatter (cell size and granulation) and 32% monocytes/macrophages.

Western blotting of TACE protein

Colonic epithelial cells or LPMNC were homogenised in 1% Nonidet P40 (Sigma) in a total volume of 50 μ l, which included the same protease inhibitor cocktail as described in the cell isolation procedure, 1 mM EDTA and phenyl methyl sulphonyl fluoride (Sigma). After incubation for 60 minutes at 0°C, undissolved membranes were removed by centrifugation and the supernatant was used for SDS electrophoresis. In order to ensure sufficient amounts of protein, cells were pooled from 2–5 individual patients. Total protein (50 μ g) was separated by electrophoresis on a 4–12% SDS gel and transferred onto ethanol activated polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The membranes were blocked for one hour in Tris 10 mM, NaCl 0.15 M, and 0.05% Tween-20 supplemented with 2% BSA and incubated overnight at 4°C with anti-TACE antibody diluted 1:5000. After additional washes in buffer, the membrane was incubated for one hour at room temperature with horseradish

Figure 1 Sodium dodecyl sulphate electrophoresis showing hydrolysis of glutathione-S-transferase-pro-tumour necrosis factor α (GST-pro-TNF- α) by recombinant TNF- α converting enzyme (TACE) or detergent extract of cell membranes from human colonic mucosa. Bands were visualised by western blotting using antibodies against GST (A) or TNF- α (B, C). Lane 1: recombinant TACE, t=0 minutes; lane 2: recombinant TACE, t=60 minutes; lane 3: recombinant TACE, t=60 minutes + inhibitor (EDTA 5 mM (A, B), CH4474 1 μ g/ml (C)); lane 4: detergent extract, t=0 minutes; lane 5: detergent extract, t=60 minutes; lane 6: detergent extract, t=60 minutes + inhibitor (EDTA 5 mM (A, B), CH4474 1 μ g/ml (C)); lane 7: purified standards. Similar results were obtained in two further independent experiments.

peroxidase conjugated antigoat rabbit antibody (Dako) diluted 1:20 000. Detection was performed with ECL western blotting reagents (Amersham Pharmacia Biotech) following the manufacturer's instructions. Gels were photographed using a Polaroid camera designed for the purpose (Amersham, UK). In control experiments the TACE antibody was incubated for two hours at room temperature with an epitope mimicking blocking peptide prior to incubation with membrane protein.

RESULTS

Demonstration of TACE activity in colonic biopsies using a recombinant pro-TNF- α assay and an unlabelled oligopeptide hydrolysis assay

Figure 1 (A, B) shows that detergent extract of cell membranes isolated from normal colonic mucosa cleaved a recombinant GST-pro-TNF- α substrate into a GST fragment and a TNF- α fragment, as identified by antibodies and standards. The GST fragment was sometimes seen as a double band, probably due to the action of other proteases. Similar results were obtained when recombinant TACE was used to cleave the substrate, and both processes were sensitive to inhibition with EDTA (fig 1A, B) and CH4474, a TACE/MMP inhibitor (fig 1C). To determine the precise cleavage site of pro-TNF- α by TACE-like activity in detergent extracts, an assay based on cleavage of an unlabelled synthetic peptide mimicking the known pro-TNF- α cleavage site was devised. As shown in fig 2 (A, B), the oligopeptide substrate was cleaved by detergent extracts yielding two products identified by HPLC. Also, this process was inhibited by EDTA and CH4474 (fig 2C, D). The molecular weight of the resulting peptides produced by cleavage of the model substrate deviated <2% from predicted. This indicates that cleavage occurred between Ala(76) and Val(77) in the corresponding pro-TNF- α sequence. Deviation should be >10% if cleavage shifts one amino acid.

Characterisation of TACE activity in human colonic mucosa using a dnp labelled oligopeptide hydrolysis assay

A modified assay allowing detection at 340 nm was required to study the effect of additional protease inhibitors, as some of these interfered with HPLC detection of unlabelled peptide breakdown due to high absorbance in the relevant concentration ranges (data not shown). The reaction was linear for both

Figure 2 Hydrolysis of an unlabelled oligopeptide substrate mimicking pro-tumour necrosis factor α (pro-TNF- α) cleavage by detergent extracts of cell membranes from human colonic mucosa. Substrate (S) hydrolysis was analysed by reverse phase high pressure liquid chromatography and the cleavage products (P1, P2) were identified by synthetic standards (A). (B) Magnification of product formation. (C, D) Inhibition by EDTA (5 mM) and CH4474 (1 μ g/ml). Similar results were obtained in five further independent experiments.

the amount of detergent extract added and for time up to one hour (data not shown). As summarised in fig 3, EDTA and CH4474 also inhibited cleavage of the dnp labelled oligopeptide, and a representative chromatogram of the effect of CH4474 is shown in fig 4. Another zinc chelating agent, phenanthroline, and BB94, a MMP/TACE inhibitor, had similar effects (fig 3). Surprisingly, TLCK also inhibited peptide cleavage but other serine protease inhibitors, such as leupeptin, had no effect (data not shown). TLCK not only inhibited enzyme activity in detergent extract but also the effect of recombinant TACE on GST-pro-TNF- α cleavage (fig 5). Phenyl methyl sulphonyl fluoride, bacitracin, and glycerol up to 20% had no effect (data not shown). The same was true for trocade, a broad spectrum MMP inhibitor, which failed to inhibit peptide hydrolysis at concentrations (5 μ M) known to inhibit several MMPs, but not TACE (fig 3).^{22 23}

Localisation and expression of TACE protein in human colonic mucosa

Using immunohistochemistry, we found that TACE appeared to be predominantly expressed in mononuclear cells of the lamina propria and in the epithelium (fig 6A, C). No significant staining was seen in control sections stained with a goat immunoglobulin (fig 6B, D). To corroborate this finding, western blotting was performed on detergent extracts of cell membranes from epithelial cells and LPMNC isolated from normal human colonic biopsies. The full length proform as well as the active form of TACE with molecular weights of about 110 kDa and 85 kDa, respectively, were identified in both cell types (fig 7). The proform appeared to consist of at least two bands, which may be due to differences in glycosylation, as reported previously.^{27 28} Immunoreactivity disappeared when the primary antibody was preincubated with an epitope specific blocking peptide.

Measurement of TACE activity in colonic mucosa from patients with IBD

The dnp labelled oligopeptide assay was used to measure TACE activity in IBD patients because sample interference was

Figure 3 Effect of protease inhibitors on tumour necrosis factor α (TNF- α) converting enzyme (TACE) activity in detergent extracts of cell membranes from human colonic mucosa, as measured by hydrolysis of a dinitrophenol labelled oligopeptide substrate mimicking the pro-TNF- α cleavage site. TACE activity was quantitated by high pressure liquid chromatography and expressed relative to protein concentration. Mean values of three individual experiments of material pooled from individual patients are shown. Samples contained dimethylsulphoxide (DMSO) 0.05% (*) when necessary for solubilisation of the inhibitors. Controls with (*) and without DMSO are shown. TLCK, N α -p-tosyl-L-lysine chloromethyl ketone.

Figure 4 Hydrolysis of a dinitrophenol (dnp) labelled peptide substrate mimicking the pro-tumour necrosis factor α (pro-TNF- α) cleavage site by detergent extracts of cell membranes from human colonic mucosa. Substrate (S) hydrolysis was analysed by reverse phase high pressure liquid chromatography and the dnp labelled cleavage product (P) was identified by synthetic standard (A). (B) Product formation was inhibited by CH4474 (1 μ g/ml).

avoided, which simplified analyses. The median TACE activity level was significantly increased in patients with ulcerative colitis (118 AU/mg, range 67–337, n=18) compared with controls (79 AU/mg, range 60–130, n=12) ($p<0.01$). Breakdown according to the grade of clinical activity showed that the median TACE level was higher in those with clinically active ulcerative colitis (147 AU/mg, range 73–337, n=12) compared with controls ($p<0.01$) but not in those with inactive disease (94 AU/mg, range 67–123, n=6) (NS). In contrast, the median TACE level in patients with Crohn's disease (81 AU/mg, range 50–166, n=10) did not differ from that in controls (fig 8), irrespective of disease activity (NS). There was no overall difference in median TACE levels between samples obtained from endoscopically normal areas (106 AU/mg, range 77–158, n=18) and areas with macroscopically active Crohn's disease

Figure 5 Sodium dodecyl sulphate (SDS) electrophoresis showing hydrolysis of glutathione-S-transferase-pro-tumour necrosis factor α (GST-pro-TNF- α) by recombinant TNF- α converting enzyme (TACE). Samples were analysed on continuous SDS polyacrylamide gels (20%) using silver staining. Lane 1: GST-pro-TNF- α substrate; lane 2: recombinant TACE, $t=0$ minutes; lane 3: recombinant TACE, $t=60$ minutes; lane 4: recombinant TACE, $t=60$ minutes + N α -p-tosyl-L-lysine chloromethyl ketone (TLCK 10 μ g/ml). Similar results were obtained in two further independent experiments.

or ulcerative colitis disease (90 AU/mg, range 70–136, $n=10$) (NS). However, separate analysis of paired biopsies from five patients with ulcerative colitis and one with Crohn's disease showed that median TACE levels were slightly higher in endoscopically inflamed areas (77 AU/mg, range 66–144) compared with normal mucosa (65 AU/mg, range 27–106) ($p<0.05$).

DISCUSSION

In this study, we found that detergent extracts of cell membranes from normal human colonic mucosa cleaved a full length pro-TNF- α substrate and released TNF- α . As release of TNF- α was sensitive to both EDTA and a TACE/MMP inhibitor,^{22,23} we concluded that biopsies contained TACE-like activity (fig 1). Next, we found that detergent extracts also cleaved a synthetic peptide mimicking the hydrolysis site for

Figure 7 Western blotting of tumour necrosis factor α (TNF- α) converting enzyme (TACE) protein immunoreactivity in detergent extract of membranes from intact biopsies (lane A), isolated epithelial cells (lane B), or purified lamina propria mononuclear cells (lane C). The arrows indicate the proform (p) and active (a) forms of TACE. Lanes D and E show the absence of immunoreactivity when an epitope mimicking blocking peptide was present during incubation. The TACE antibody was the same as that used in fig 6. Similar results were obtained in three independent experiments.

TACE in the pro-TNF- α sequence at amino acids 76–77. From these data we concluded that biopsies contained TACE-like activity that cleaves a model peptide for the human TNF- α precursor at the correct site (fig 2). Finally, we used a dnp labelled oligopeptide assay to study the effect of inhibitors. The effect of these was as predicted (fig 3)^{19,29–31} with the

Figure 6 Immunohistochemistry of tumour necrosis factor α (TNF- α) converting enzyme (TACE) protein expression in normal human colonic mucosa. (A) ($\times 20$) and (C) ($\times 80$) show that TACE was widely expressed in lamina propria mononuclear cells and in crypt epithelial cells. (B) ($\times 20$) and (D) ($\times 80$) show the corresponding background staining when the primary antibody was substituted with unspecific goat immunoglobulins. Examples of cells regarded as positive for TACE are indicated by arrows (C). Similar results were obtained in two independent experiments.

Figure 8 Tumour necrosis factor α (TNF- α) converting enzyme (TACE) activity in detergent extracts of cell membranes from colonic mucosa from patients with ulcerative colitis (UC) or Crohn's disease (CD) and healthy controls. TACE activity is expressed as specific activity (SA) in arbitrary units per mg of protein based on high pressure liquid chromatography analysis of hydrolysis of a dinitrophenol labelled oligopeptide assay substrate mimicking the pro-TNF- α cleavage. Horizontal lines indicate median values. ** $p < 0.01$.

exception of TLCK, which may be regarded as an unspecific TACE inhibitor (fig 5). Purified matrilysin, stromelysin, collagenase, the gelatinases, as well as membrane type (MT)-MMPs have been shown to cleave pro-TNF- α to soluble TNF- α in vitro.^{19–32} It is therefore important that trocade, an inhibitor of several MMPs, but not TACE itself, was ineffective even at concentrations fivefold above those required for inhibition of stromelysin-1 (MMP-3) and many fold above those needed to inhibit several other MMPs.^{22–23} Taken together, our results show that an enzyme with functional properties similar to TACE is present in human colonic mucosa and presumably responsible for TNF- α processing.

Next we used immunohistochemistry to localise TACE protein in human colonic mucosa. TACE protein was, as expected, widely expressed in LPMNC^{10–33} but immunoreactivity was also present in the epithelium (fig 6). These data were confirmed by western blotting of cell membrane protein from purified human colonic epithelial cells or LPMNC showing that both cell fractions expressed the proform as well as the active form of TACE²⁸ (fig 7). The latter observation is consistent with the recent demonstration of a correlation between TACE and CD68 antigen expression in synovial tissue cells.³³ The presence of TACE protein in human colonic epithelial cells has not been described previously but this is not surprising in light of the ubiquitous expression of this sheddase in a variety of non-immune human cells, including endothelial cells, small muscle cells, and chondrocytes.^{10–13} Interestingly, *tace* ^{Δ 20/ Δ 20} (knockout) mice are characterised by several cellular shedding defects and display a range of epithelial abnormalities, including blunted small bowel villi and pseudostratification of the mucosal epithelium.³⁴ Further study is required to characterise the function of TACE protein expressed in human colonic epithelial cells.³⁵

Functional TACE activity was increased in patients with ulcerative colitis, particularly in those with clinically active disease (fig 8). This accords with our previous demonstration of a modest but significant twofold increase in mucosal TACE mRNA levels in an entirely different sample of patients with ulcerative colitis.¹⁵ Hence both mRNA expression and TACE activity appear to be elevated in ulcerative colitis patients. Moreover, the level of enzyme activity was somewhat higher in

areas with endoscopically active inflammation when patients were their own controls. Interestingly, similar changes have recently been demonstrated in arthritis affected cartilage as well as in synovial tissue of patients with rheumatoid arthritis.^{33–36} TACE activity was also present in Crohn's disease mucosa but not increased compared with controls (fig 8). This contrasts our previous finding of a correlation between TACE mRNA expression and Crohn's disease activity¹⁵ and one possibility is that inactivation of TACE activity has been catalysed in Crohn's disease mucosa by other MMPs as part of a self-regulatory mechanism similar to that described for MT1-MMP-MMP-2 interactions.³⁷ Increased mRNA expression, but not increased enzyme activity, would be detected in such a setting. Recently, phorbol 12-myristate-13-acetate, a potent inducer of shedding, has been shown to downregulate TACE expression in vitro, and since the effect was inhibited by a MMP inhibitor, shedding processes appear to be under feedback control.³⁸ Whether a similar downregulatory mechanism is operative in vivo in Crohn's disease mucosa, but not in ulcerative colitis, remains purely speculative at this stage.

Inhibition of TACE activity strongly downregulates T cell release of soluble TNF- α in vitro, which may lead to accumulation of the membrane bound precursor^{10–30} thought to be biologically active.³⁹ Shedding of downregulatory soluble TNF- α receptors is also compromised and TACE/MMP inhibition may in theory carry a proinflammatory potential.¹³ However, a recent study showed that oral administration of a potent MMP/TACE inhibitor to healthy human subjects strongly reduced lipopolysaccharide induced TNF- α release without influencing the level of monocyte bound TNF- α .⁴⁰ Synthetic MMP inhibitors have been shown to downregulate inflammation in rat models of arthritis⁴¹ and IBD^{42–43} and to protect against endotoxin shock in mice by preventing TNF- α processing.⁴⁴ Although the MMP inhibitors tested have a variable ability to block sheddase enzyme activity, the data make it less likely that this class of drugs have a general proinflammatory effect in vivo. Shedding of L-selectin and transforming growth factor α is also compromised in TACE deficient mice³⁸ and MMP inhibitors reduce the release of the soluble Fas ligand by T cells in vitro,⁴⁵ but the exact clinical significance of these effects remains unclear.

In conclusion, we have shown that functional TACE activity is ubiquitously expressed in normal or Crohn's disease colonic mucosa and upregulated in ulcerative colitis. Secretion of TNF- α is definitively increased in IBD^{26–47–49} and drugs targeting the final stage of TNF- α processing may therefore be of benefit. As TACE is structurally related to the family of MMPs, which have been strongly implicated as final mediators of TNF- α -induced proteolytic gut mucosal damage,^{16–18–30} our data may be helpful in creating a rationale for therapeutic intervention in ulcerative colitis using specifically tailored synthetic MMP inhibitors that also target TACE.

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