Isolated guinea pig gastric chief cells express tumour necrosis factor receptors coupled with the sphingomyelin pathway

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Abstract

The tumour necrosis factor alpha (TNF), has been implicated in the pathogenesis of non-steroidal anti-inflammatory drug (NSAID) induced gastropathy and *Helicobacter pylori* induced gastritis. Both conditions are characterised by high plasma pepsinogen concentrations, which are thought to reflect an increased rate of enzyme release by the pepsinogen secreting (chief) cells. The mechanisms responsible for this cell dysfunction are unknown. This study investigates whether chief cells express TNF receptors and, if so, whether their activation results in cell death. Immunohistochemical studies conducted with monoclonal antibodies (mAbs) directed against two TNF receptor associated proteins of 55 kDa (TNF-R1) and 75 kDa (TNF-R2) showed that TNF binding sites were expressed in approximately 100% gastric chief cells. Western blot analysis of whole chief cell lysates probed with the TNF-R1 and TNF-R2 mAbs gave two distinct bands of 55 and 75 kDa in the immunoprecipitate. Incubating chief cells with TNF caused concentration and time dependent cell death, which was prevented by pretreating the cells with anti-TNF receptor mAbs. Exposing the cells to TNF reduced sphingomyelin content by 25%. Sphingomyelinase (10^{-6} to 10^{-2} IU/ml) mimicked the effect of TNF in that it provoked a concentration and time dependent reduction in chief cell viability and increased pepsinogen release. In conclusion, gastric chief cells express two TNF receptors partially linked to the sphingomyelin pathway. TNF induced chief cell dysfunction might be responsible for the high plasma pepsinogen concentrations seen in patients with NSAID gastropathy or *H pylori* induced gastritis.

(Gut 1996; 38: 182-189)

Keywords: cytokines, *Helicobacter pylori*, non-steroidal anti-inflammatory drugs, apoptosis, gastritis, second messengers.

Tumour necrosis factor alpha (TNF) is a multifunctional cytokine, mainly produced by activated macrophages, which signal a variety of cellular responses including cytotoxic, antiviral, inflammatory, immunoregulatory, and proliferative activities.¹⁻³ TNF signalling is initiated by interaction of TNF with distinct

cell surface receptor molecules. Two of these receptors, of 55 kDa (p55 or TNF-R1) and 75 kDa (p75 or TNF-R2), have recently been identified and cloned.⁴⁻¹⁰ In some systems, TNF-R1 is thought to signal both cytotoxic and proliferative responses, while the role of TNF-R2 in signalling cytotoxicity remains controversial.⁴⁻¹⁰

TNF has been implicated in the pathogenesis of several gastrointestinal affections including non-steroidal anti-inflammatory drug (NSAID) induced gastropathy, *Helicobacter pylori* induced gastritis, necrotising enterocolitis, and chronic inflammatory bowel diseases.¹¹⁻¹⁵ TNF has been shown to upregulate the expression of cell surface adhesion molecules on both circulating neutrophils (PMNs) and endothelial cells and so favour the binding of PMN to vessels at sites of inflammation.¹⁶ Although the mechanism of NSAID induced gastric injury is generally believed to be related to the ability of these agents to inhibit gastric prostaglandin generation, evidence is accumulating that PMN adherence to the vascular endothelium is one of the early and pivotal events.¹⁶ We have shown that indomethacin increases TNF plasma concentrations and that pretreating rats with substances that inhibit TNF synthesis or release prevents NSAID induced mucosal damage.^{13,14} *H pylori* induced active antral gastritis also depends on PMN recruitment and activation.¹⁷ *H pylori* secretes potent PMN chemotaxins and substances capable of activating peripheral blood monocytes to produce IL-1 β and TNF.¹⁸ Moreover, short-term cultures of *H pylori* infected gastric mucosa cells have been found to produce IL-6, IL-8, and TNF and the content of IL-8 and TNF mRNA had been shown to be considerably increased in antral biopsy specimens obtained from *H pylori* infected patients.¹⁷⁻¹⁹

Whether inflammatory cytokines affect gastric epithelial cell functions is unknown.²⁰ However, the finding that IL-1 β inhibits basal and pentagastrin stimulated acid and pepsin secretion suggests that both parietal and peptic (chief) secreting cells might be modulated by cytokines.^{21,22} *H pylori* induced antral active gastritis and NSAID induced gastropathy are characterised by raised plasma pepsinogen concentrations, which are thought to reflect an increased rate of zymogen release into the circulation.²³ Derangement of pepsinogen release is of pathogenetic relevance because, however activated, intramucosal zymogens may participate in the genesis of acute mucosal

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Accepted for publication
28 July 1995

lesions.²³ This study was designed to investigate whether TNF is able to modulate pepsinogen release from isolated chief cells. As TNF is a powerful cytotoxic agent we investigated whether chief cells express TNF receptors whose activation leads to cell dysfunction or programmed cell death, or both. As in other systems, TNF effects are mediated by a positive coupling with the sphingomyelin pathway,²⁴⁻²⁷ we also investigated whether activation of TNF receptors stimulates intracellular sphingomyelin degradation.

Methods

Materials

Male guinea pigs (200–250 g) were obtained from Morini (Monza, Italy). N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES), serum bovine albumin fraction V (BSA), soybean trypsin inhibitor, collagenase (type I), carbamylcholine, ethylene glycol-bis (B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), EDTA, silica gel G plates, sodium dodecyl sulphate (SDS), polyacrylamide gel for electrophoresis (PAGE), aprotinin, polyoxyethylenesorbitan monolaurate (TWEEN 20), phenylmethylsulphonyl fluoride (PMSF), sphingomyelin, phosphatidylcholine, phosphatidylethanoamine, sphingomyelinase (from *Staphylococcus aureus*) (SMase), and lactate dehydrogenase (LDH) assay kit were from Sigma Chemical (St Louis, MO); Percoll from Pharmacia (Milan, Italy); essential aminoacid mixture and 1% essential vitamin mixture from Gibco (Milan, Italy); fetal calf serum (FCS) from Flow (Milan, Italy), fluorescein isothiocyanate (FITC) labelled goat antimouse IgG from Kirkegaard and Perry Laboratories (Gaithersburg, MD). C₂ ceramide was from Biomol Research Laboratories (Plymouth Meeting, PA). Human recombinant TNF 1×10⁷ IU/mg, IgG 1 mouse monoclonal antibodies (mAb) against the human TNF-R1 (Htr-9) and the human TNF-R2 (Utr-1), were generously provided by Dr M Brockhaus and Dr L Loetscher (Hoffmann-La Roche, Basle, Switzerland).⁴⁻⁶

Chief cell preparation

Chief cells from guinea pig stomach were prepared as previously described.^{28,29} Briefly, guinea pigs were killed by cervical dislocation, the stomach removed, opened along the lesser curvature, placed with mucosal surface down on a wax tray, and the mucosa separated from the muscular layer by injecting incubation buffer with a 35 ml syringe. The mucosa was removed, minced, placed in 40 ml of incubation buffer containing 40 mg type I collagenase, gassed with 100% O₂, and incubated for 40 minutes at 37°C in a shaking water bath (160 oscillations/min). After incubation, the mixture was filtered through a 210 µm Teflon mesh, centrifuged to remove the collagenase, resuspended in 40 ml of standard incubation buffer containing 400 µl 200 mM EGTA, gassed with 100% O₂, and incubated at 37°C

for 15 minutes. Cells were then washed to remove the EGTA, filtered through a 70 µm mylon mesh, resuspended in 2 ml incubation buffer, mixed with 10 ml Percoll solution, and centrifuged at 16 000 rpm in a JC21 Beckman centrifuge at 4°C for 15 minutes. The density of the resulting Percoll gradient was controlled by suspending coloured density marker beads in 2 ml of standard incubation solution and processing this solution in a centrifuge tube containing gradient material identical to that used for cell suspension. After centrifugation chief cells form a layer at densities between 1060 and 1080 g/ml. This method yields a cell population that is approximately 95% chief cells and 5% other cells.^{28,29} Chief cells were suspended in a standard incubation solution containing 24.5 mM HEPES, 120 mM NaCl, 7.2 mM KCl, 1.5 mM Ca₂Cl₂, 0.8 mM MgCl₂, 2.6 mM KH₂PO₄, 14 mM glucose, 6 mM Na pyruvate, 6 mM glutamate, 7 mM fumarate, 2 mM glutamine, 0.1% (wt/vol) trypsin inhibitor, 0.1% (wt/vol) albumin, 1% (vol/vol) essential aminoacid mixture, and 1% (vol/vol) essential vitamin mixture. The pH was 7.4 and all incubations were performed with 100% O₂ as the gas phase.

Immunohistochemistry

Serial cryostat sections were cut at 5–8 µm from snap frozen stomach specimens (approximately 2.5×1.0×0.3 cm), air dried on poly-L-lysine coated slides for two to three hours, then fixed in acetone at room temperature for 10 minutes. Sections from the same tissue blocks were also stained with haematoxylin and eosin for histological comparison. Paraffin wax sections from formalin fixed tissue specimens were also prepared and stained with haematoxylin and eosin for standard morphological evaluation. Immunostaining with anti-TNF receptor mAb Utr-1 and Htr-9 (30 µg/ml) was performed using a standard alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (DAKO APAAP KIT SYSTEM 40 K670, Dako, Carpinteria, CA).³⁰ Antibody binding was visualised with naphthol AS-MX phosphate Fast Red substrate chromogen. Endogenous alkaline phosphatase activity was blocked by including levamisole in the substrate solution. The sections were counterstained with haematoxylin and eosin mounted in SUPERMOUNT (BioGenex, San Ramon, CA). Negative controls for each experiment were performed using mAb unreactive with guinea pig determinants (Ber-H2), followed by the appropriate fluorochrome conjugated goat antimouse antibody.

Isolated chief cells were centrifuged for immunostaining at a concentration of 2×10⁵ cell/ml. Cytospins were air dried overnight and fixed in acetone at room temperature for five minutes and then incubated for 30 minutes with Htr-9 or Utr-1 mAb (30 µg/ml) at 1:20 dilution. After three washes in TRIS buffer 0.1 mol/l (pH 7.6), the smears were incubated with rabbit antimouse immunoglobulin for 30 minutes, rinsed, and incubated with the APAAP complex for 30 minutes. Negative

Figure 1: Immunostaining of U-937 and isolated chief cell cytopins. Negative controls were obtained by incubating U-937 cells (A) and chief cells (B) with a mouse mAb that recognises human monocyte/macrophage associated antigen (anti-Ber H2). (C) and (D), staining of TNF-R1 (Htr-9 mAb) and TNF-R2 (Utr-1 mAb) on U-937 cells. (E) and (F), staining of TNF-R1 (Htr-9 mAb) and TNF-R2 (Utr-1 mAb) on chief cells. APAAP technique, haematoxylin counterstaining; $\times 100$.

controls for each experiment were performed using mAb unreactive with guinea pig determinants (Ber-H2), followed by the appropriate fluorochrome conjugated goat antimouse antibody.

Immunoblotting precipitation of TNF-R

Isolated chief cells and U-937 cells were washed twice with PBS and lysed in 1 ml/ 1×10^7 cells lysis buffer (a mixture of 250 mM NaCl; 25 mM TRIS-HCl; 5 mM EDTA; 1% Triton X-100; 2 mg/ml aprotinin; 1 mM PMSF) for 20 minutes on ice, and then clarified in a microcentrifuge at 4°C. The cell lysates were pretreated with 25 μ l/ml fetal calf serum, incubated overnight with 40 μ M/ml Htr-9 or Utr-1, added to protein A-Sepharose beads, and incubated for 90 minutes at 4°C. Immunocomplexes were washed three times in a washing buffer that had the same composition as the lysis buffer, but 0.1% Triton $\times 100$, placed on a rotatory mixer at 4°C for five minutes, and resuspended in 50 ml of SDS-PAGE loading buffer. Antigen was eluted by heating the tubes to 100°C for three minutes, samples were run in a 12.5% SDS-PAGE, and

then transferred to nitrocellulose membranes. The blots were incubated for one hour in PBS containing 0.1% TWEEN 20 (PBS-T) at room temperature on an orbital shaker, and excess block removed by rinsing three times in PBS-T. Blots were then incubated with appropriate dilutions of Htr-9 and Utr-1 for one hour, washed four times in PBS, and incubated for one hour with a peroxidase labelled secondary antibody. After further washes in PBS-T, blots were developed using the enhanced chemiluminescence (ECL) detection kit (ECL Western blotting kit, Amersham, Milan, Italy).³¹

Cell viability

To evaluate the effect of increasing concentrations of TNF on chief cell viability, chief cells ($2 \times 10^6/2$ ml) were incubated alone or with 5 to 2000 IU/ml TNF at 37°C for one hour and cell viability, LDH, and pepsinogen release into cell supernatants measured. The effect of time was studied by incubating chief cells ($2 \times 10^6/2$ ml) with 500 IU/ml TNF for up to three hours and cell viability, LDH, and pepsinogen release into the cell supernatants

Figure 2: Immunoblotting of TNF binding proteins from isolated chief cells and U-937 cells (positive control). Blots obtained from chief cells and U-937 cell lysates were incubated with 10 µg/ml Htr-9 or Utr-1. Immunoprecipitates from isolated chief cells are shown in lanes A and immunoprecipitates from U-937 cells in lanes B. Molecular mass markers are shown on the left. Both isolated chief cells and U-937 cell lysates precipitated two protein bands, one at 55 kDa with Htr-9 mAb (TNF-R1) and one at 75 kDa with Utr-1 mAb (TNF-R2). There was also a small precipitation band at about 75 kDa in chief cell lysates incubated with Htr-9 mAb.

determined every 15–30 minutes. Chief cell viability was assessed by adding 50 µl trypan blue solution (0.4 g/dl) to 500 µl of chief cell suspension and counting the number of stained and unstained cells within 10 minutes. LDH released from isolated chief cells was assayed in cell supernatants. For this purpose, isolated chief cells (1×10^6 cells/ml) were suspended in the standard incubation solution, from which the pyruvate was omitted, and incubated alone or with the concentrations of TNF and for the amount of time specified in Figs 4 and 5. LDH released during the incubation was determined by a spectrophotometric assay using pyruvate and NAD as substrates. LDH released into the medium was expressed as a percentage of total cellular LDH content

Figure 3: Effect of increasing concentrations of TNF on chief cell viability and LDH and pepsinogen release on isolated gastric chief cells. Data are means (SEM) of 10–12 different experiments. Each sample was both incubated and assayed in duplicate. * $p < 0.05$ by Student's *t* test and by ANOVA.

present in the chief cells at the beginning of the incubation. Pepsinogen released into the medium was assayed using acid denatured haemoglobin as substrate.³² Pepsinogen secreted during incubation was expressed as a percentage of total pepsinogen present in the chief cells at the beginning of the incubation minus the pepsinogen secreted prior to starting incubation.

Inhibition of TNF induced cytotoxicity by anti-TNF mAb

As previous studies have shown that 10 µg/ml of Utr-1 and Htr-9 antagonise the cytotoxic effect of TNF on the U-937 cell line,⁵ we first investigated whether this mAb concentration protected chief cells from TNF induced cytotoxicity and whether anti-TNF mAb in itself exerted any effect on chief cell viability. Chief cell (1×10^6) were preincubated for 15 minutes alone or with 10 µg/ml Utr-1 or Htr-9 and then reincubated alone or with 500 IU/ml TNF for one hour. To test whether the protective effect exerted by the anti-TNF mAbs was dose dependent, chief cells (1×10^6 /ml) were preincubated alone or with scaled up concentrations of Htr-9 or Utr-1 (from 100 ng/ml to 19 µg/ml) alone or in combination at 37°C for 15 minutes and then reincubated alone or with 500 IU/ml TNF for one hour and the number of trypan blue stained and unstained cells counted and LDH and pepsinogen released into cell supernatants determined.

Effects of TNF on sphingomyelin content on isolated chief cells

Chief cells (2.5×10^7) were incubated with 500 IU/ml TNF for varying periods of time at 25°C (see Fig 7). Incubations were stopped by adding 3 ml chloroform/methanol mixture (2:1, vol/vol). Lipids were extracted according to the method proposed by Folch *et al*³³ and separated by two dimensional thin layer chromatography on silica gel G plates using chloroform/methanol 30% ammonia (65:25:4, vol/vol) in the first dimension and chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5, vol/vol) in the second. Lipids were detected by exposing the plates to iodine vapours. Identification of each lipid was achieved by comparison with standards. The spots corresponding to sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine were scraped off and their phosphorus content determined according to van Veldhoven and Mannaerts.³⁴

Effect of SMase and ceramide on chief cell viability and pepsinogen release

As ceramide is the main product of sphingomyelin metabolism in mammalian cells, we tested the effect of a synthetic, cell permeable C₂-ceramide and SMase on cell viability and enzyme release. Chief cells (1×10^6 /ml) were incubated alone, with increasing concentrations of SMase (1×10^{-7} to 10^{-3} U/ml) or with C₂-ceramide at concentrations ranging from

Figure 4: Effect of length of incubation on TNF induced cytotoxicity. Chief cells were incubated alone or with 500 IU/ml TNF and viability and LDH release determined at the time shown. Data are means (SEM) of 10 experiments. Each sample was both incubated and assayed in duplicate.

10 nM to 10 μ M for 30 minutes and cell viability (trypan blue) and the LDH and pepsinogen released into chief cell supernatants assayed.

Statistical analysis

Data are reported as the mean (SEM) of the number of experiments indicated and analysed by the analysis of variance (ANOVA) and the Student's *t* test.³⁵

Results

Immunohistochemistry

Anti-TNF receptor mAbs labelled oxintic glands in the normal guinea pig stomachs. Utr-1 and Htr-9 positive cells exhibited membrane and diffuse cytoplasmic positivity and were found in the basilar two thirds of glands where chief and parietal cells are localised (data not shown). When cell suspensions were examined, neither U-937 nor chief cells cross reacted with the anti-Ber-H2 mAb (Fig 1(A) and (B)). Staining of U-937 cells with Htr-9 and Utr-1 gave a membrane and diffuse cytoplasmic positivity (Fig 1(C) and (D)). As almost 100% isolated gastric chief cells incubated with HTR-1 and UTR-1 manifested the same staining pattern (Fig 1(E) and (F)), they too expressed both TNF receptors.

Immunoblotting precipitation of TNF binding proteins

Immunoprecipitation of p55 and p75 (receptor associated proteins) obtained from whole chief cell lysates with Htr-9 and Utr-1 yielded two

distinct protein precipitate bands. Htr-9 mAb caused the precipitation of a wide protein band of 55 kDa (TNF-R1) and a small band of about 75 kDa, whereas the Utr-1 mAb precipitated only a 75 kDa (TNF-R2) protein band (Fig 2, left lanes). U-937 cells, which overexpress both TNF-R1 and R2 (positive control), exhibited a similar protein band precipitation pattern (Fig 2, right lanes).

Effect of TNF on cell viability

As TNF is cytotoxic for several cell lines, its effect on chief cell viability was investigated. Almost all chief cells, 92.3 (2.4)%, incubated alone remained viable for at least three hours in terms of their ability to exclude trypan blue and maintain intracellular LDH and pepsinogen concentrations. Basal pepsinogen and LDH release averaged 0.9 (0.3)% and 2.0 (0.4)% of total cellular enzyme content. Incubating chief cells with increasing concentrations of TNF reduced chief cell viability and increased both LDH and pepsinogen release dose dependently (Fig 3). Incubation with 500 IU/ml TNF for one hour decreased cell viability to 45.4 (2.2)% ($p < 0.05$) and triggered the release of 46.8 (2.9)% total cellular LDH ($p < 0.05$) and 21.5 (1.4)% total cellular pepsinogen ($p < 0.05$). The experiment that tested the effect of time on TNF induced cytotoxicity showed that three hour incubation with 500 IU/ml TNF decreased cell viability from 93.0 (1.7)% to 35.2 (2.7)% ($p < 0.05$) and increased LDH and pepsinogen release to 70.6 (3.0)% and 35.3 (3.2)% ($p < 0.05$) (Fig 4).

Inhibition of TNF induced cytotoxicity by anti-TNF mAb

Chief cells preincubated alone and then incubated without TNF had a cell viability of 92.2 (1.2)%; pepsinogen release averaged 1.9 (0.3)% and LDH release 2.2 (0.4)%. Incubating isolated chief cells with 10 μ g/ml Utr-1 or Htr-1 had no effect on cell viability, LDH or pepsinogen release (data not shown). When chief cells were preincubated alone, however, and then exposed to 500 IU/ml TNF for one hour, viability decreased to 62.4 (2.4)% ($p < 0.05$), and LDH and pepsinogen release increased to 42.0 (3.4)% and 25.5 (4.9)% ($p < 0.05$). Preincubation with 10 μ g/ml Htr-9 or Utr-1 protected chief cells from the cytotoxic effect of TNF (Fig 5, left panel). In chief cells preincubated with 10 μ g/ml Utr-1 or Htr-9 and then exposed to 500 IU TNF LDH release decreased to 3.5 (1.0) and 4.1 (0.8) ($p < 0.05$), and pepsinogen release to 2.0 (0.7) and 2.3 (1.0) ($p < 0.05$). As shown in Fig 5 (right panel), the protective effect each anti-TNF mAb exerted on TNF induced chief cells necrosis was concentration dependent ($EC_{50} = 1 \mu$ g) and no further protection was obtained when the two mAb were combined.

Effect of TNF on sphingomyelin content on isolated chief cells

Incubating chief cells with TNF reduced

Figure 5: Upper panel. Inhibition of TNF induced cytotoxicity by anti-TNF-R1 (Htr-9) and anti-TNF-R2 (Utr-1) mAbs. Chief cells were preincubated alone or with 10 μg/ml Htr-9 or Utr-1 and then reincubated alone (controls) or with 500 IU/ml TNF. Data are means (SEM) of seven experiments. Each sample was both incubated and assayed in duplicate. * $p < 0.05$. Lower panel. Htr-9 and Utr-1 determined a concentration dependent inhibition of TNF induced cytotoxicity with an EC_{50} of 1 μg/ml. No further protection was obtained by combining the two mAbs together. * $p < 0.05$ versus chief cells incubated with TNF alone.

sphingomyelin content time dependently from 11.0 (1.9) to 8.3 (1.1) pM/ 2.5×10^7 cells ($p < 0.05$) after 30 minutes of incubation. The reduction was already significant after five minutes and lasted for 30 minutes (Fig 6). As there was no modification in either phosphatidylcholine or phosphatidylethanolamine

content (data not shown) the degradation effect was sphingomyelin specific. The question of whether one or both TNF-R are required to activate sphingomyelin hydrolysis was considered by preincubating chief cells with each anti-TNF-R mAb, separately. As the Utr-1 mAb failed to block the TNF induced sphingomyelin degradation (from 11.5 (1.1) to 8.5 (1.4) pM/ 2.5×10^7 cells, $p < 0.05$, mean of five experiments), whereas Htr-9 prevented it completely (from 11.0 (1.5) to 10.5 (1.3) pM/ 2.5×10^7 cells; mean of four experiments), the sphingomyelin pathway seems to operate through TNF-R1.

Effect of SMase and ceramide on chief cell viability and pepsinogen release

Incubating chief cells with SMase reduced chief cell viability and increased LDH and pepsinogen release into cell supernatants (Fig 7) in a concentration dependent manner. Maximal cytotoxic effect was seen at 1 mU/ml SMase. Cell viability fell from 90.1 (2.0) to 53.5 (2.1)% (mean of five experiments) after one hour incubation with 1 mU/ml SMase and to 42.5 (4.5)% after three hours, while LDH and pepsinogen release rose from 2.7 (1.5) and 1.3 (0.6)% to 37.9 (5.2) and 21.8 (2.7)% ($p < 0.05$) after one hour and to 54.6 (6.0) and 31.6 (3.2)% ($p < 0.05$) after three hours. C2-ceramide had a less pronounced effect: 10 μM reduced cell viability from 91.7 (2.0) to 74.8 (5.3)% and released 21.9 (3.2)% of cellular LDH and 11.5 (2.1)% of total cellular pepsinogen.

Discussion

The results of our investigation provide evidence that isolated guinea pig gastric chief cells express TNF receptors and that their activation leads to cell death by triggering a second messenger system partially linked to the sphingomyelin pathway. In terms of concentration and exposure time required, the

Figure 6: Induction of sphingomyelin turnover in response to TNF in isolated chief cells. Chief cells (2.5×10^7) were incubated for a variable amount of time with 500 IU TNF and sphingomyelin content assayed. Data are means (SEM) of five experiments. Each sample was both incubated and assayed in duplicate. * $p < 0.05$.

Figure 7: Effect of SMase on chief cell viability (top panel) and LDH and pepsinogen release (bottom panel). Chief cells (1×10^6 /ml) were incubated with concentrations of SMase ranging from 10^{-6} to 10^{-3} U/ml for 30 minutes and viability (trypan blue) and LDH and pepsinogen release determined. Data are means (SEM) of five experiments. Each sample was both incubated and assayed in duplicate. * $p < 0.05$.

cytotoxicity induced by TNF in isolated chief cells agrees well with the cytotoxic effect TNF exerts on neoplastic and non-neoplastic TNF sensitive cell lines.¹⁻¹⁰ Immunohistochemical studies showed that TNF receptors are normally expressed on glandular epithelial cells, mainly in the basilar two third of the gastric glands. This distribution agrees with immunohistochemical studies showing that anti-pepsinogen positive (chief) cells are mainly located in the basilar region of guinea pig gastric glands.²⁸ Immunohistological studies carried out on a pure population of isolated chief cells showed that almost 100% of the cells stained for the anti-TNF-R mAbs and the finding was confirmed by western blot analysis. The Utr-1 mAb selectively precipitates a protein band of 75 kDa, which corresponds to TNF-R2, while the Htr-9 mAb precipitated a broad protein band of 55 kDa, which corresponds to the TNF-R1, and a narrow band of about 75 kDa.¹⁻¹⁰ The fact that the extracellular domains of the two receptors share 28% homology¹ may account for the apparent lack of Htr-9 specificity.

Incubating chief cells with TNF caused concentration and time dependent cell necrosis. As pretreating chief cells with mAbs directed against TNF-R1 or TNF-R2 prevented TNF induced cytotoxicity, both receptors may function as a signal recognition for TNF. Despite the many investigations conducted to identify the mechanisms implicated in TNF induced cytotoxicity, there is still doubt regarding the specific role of the two TNF receptors. TNF-R1 is thought to mediate many of the functions of TNF, including cytotoxicity.⁸⁻¹⁰ Experiments with polyclonal and monoclonal anti-human TNF-R1 mAbs with agonistic activity have shown that TNF-R1 signals cytotoxicity in U-937, HeLa, SV80, FS11, and HEP-2 cells.¹⁻¹⁰ Moreover, Tartaglia *et al*^{8,9} have recently identified an approximately 80 aminoacid region within the TNF-R1 intracellular domain, close to the C terminus of the receptor (death domain), which is necessary and sufficient for signalling cytotoxicity in L-929 cell clones.

Although information on the role of TNF-R2 is less conclusive, activation of this receptor by specific TNF-R2 agonist mAbs has also been found to promote a cytotoxic effect.¹⁰ mAb based studies are difficult to interpret, but the fact that both TNF receptor antagonists completely prevented TNF induced chief cell cytotoxicity, while the combination of the two mAbs did not increase chief cell protection, suggests that both the p55 and p75 molecular components of the TNF receptors participate in the transduction of cytotoxic signals in this cellular system.

TNF is thought to engage the sphingomyelin pathway to affect signal transduction by its receptors.²⁴⁻²⁷ This pathway is triggered when plasma membrane sphingomyelin is hydrolysed to ceramide through the action of a specific SMase.²⁶ Ceramide serves as a second messenger, stimulating serine/threonine ceramide activated protein kinase to transduce the cytokine signals, in part through mitogen activated protein kinase and transcription factors such as NF- κ B.²⁶ TNF induced sphingomyelin degradation was coupled only with the p55 TNF receptor, as shown by the fact that preincubating chief cells with Htr-9, but not with Utr-1, prevented TNF induced sphingomyelin hydrolysis. As TNF did not modify the concentrations of other choline containing lipids, including phosphatidylcholine and phosphatidylethanolamine, the effect seems to be specific.

When the role of the sphingomyelin pathway in mediating TNF induced chief cell toxicity was further investigated by incubating cells with exogenous SMase and C₂-ceramide, SMase largely reproduced the TNF effect. It caused a concentration and time dependent reduction of chief cell viability. The maximal cytotoxic effect was seen at the concentration of 1 mU/ml and increased with the length of incubation. Although C₂-ceramide also proved cytotoxic, its effect was less pronounced.²⁴⁻²⁷ Our finding that both anti-TNF receptor mAbs prevented the TNF mediated cytotoxic effect, but only TNF-R1 was coupled with

sphingomyelin degradation, activation of the sphingomyelin pathway, does not seem sufficient to account for the TNF effects.³⁶ Taken together our data suggest either that TNF-R1 is able to couple with different effector systems or, perhaps more likely, that activation of SMase occurs 'downstream' from the primary receptor activation response.

The effect TNF exerts on isolated gastric chief cells is of potential interest, as intramucosal peptic activity seems to participate in the genesis of acute mucosal lesions, as shown by the fact that it is greater in aspirin treated rats and higher in areas of erosions than normal gastric mucosa.²³ Moreover aspirin has been found to activate intramucosal zymogens by increasing hydrogen ion back diffusion.²³ NSAID gastropathy and *H. pylori* induced active gastritis are clinical conditions characterised by increased mucosal TNF concentrations and high plasma pepsinogen concentrations. Although in vitro studies are difficult to extrapolate to clinical situations, our results strongly suggest that gastric chief cell damage caused by locally produced TNF, or other cytokines or inflammatory substances,³⁷ or both, increases pepsinogen release into the gastric mucosa and that enzyme reabsorption into the blood stream triggers the increase in plasma pepsinogen concentrations seen in patients with NSAID gastropathy or active antral gastritis.

In conclusion, this study provides the first evidence that untransformed gastric chief cells express two types of TNF receptors and that their activation directly induces chief cell dysfunction and death. The effect could be responsible for increasing pepsinogen release into the gastric mucosa and so explain the high pepsinogen concentrations seen in patients with NSAID gastropathy or *H. pylori* induced antral gastritis.

We thank Federici Barbara for technical help and Judy Etherington for help with the manuscript. The investigation was in part supported by a grant from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST), Rome.

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