INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 23: 41-48, 2009

SPANDIDOS PUBLICATIONS

TACE inhibition amplifies TNF-α-mediated colonic epithelial barrier disruption

THOMAS FRÉOUR[1], ANNE JARRY[1], KALYANE BACH-NGOHOU[1], THOMAS DEJOIE[1], CHANTAL BOU-HANNA[1], MARC G. DENIS[2], JEAN-FRANÇOIS MOSNIER[1], CHRISTIAN L. LABOISSE [1] and DAMIEN MASSON[1]

[1]INSERM U539, Faculté de Médecine, F-44035 Nantes; [2]CNRS UMR 6061, Institut de Génétique et Développement, Université Rennes 1, 35043 Rennes, France

Received August 19, 2008; Accepted September 29, 2008

DOI: 10.3892/ijmm_00000099

Abstract. Inflammatory bowel diseases (IBD) are characterized by tumor necrosis factor α (TNF-α)-mediated epithelial barrier disruption. TNF-α production and the bioavailability of its receptors on the cell surface are regulated by TACE (TNF-α converting enzyme), a pleiotropic metalloprotease also known as ADAM17, and its specific inhibitor TIMP3. We therefore examined ADAM17 and TIMP3 expression in human intestinal epithelial cells (IEC) using immunohistochemistry on tissue microarrays and real-time PCR on preparations of IEC isolated from human normal and IBD colon. The effects of TACE inhibition by TIMP3 or a pharmacological inhibitor were assessed in inflammatory conditions on a TIMP3-deficient colonic cell line HT29-Cl.16E. Both TACE and TIMP3 were found to be constitutively expressed by intestinal epithelial cells in the normal and inflammatory human intestinal barrier. In the TIMP3-deficient cell line, the addition of recombinant human TIMP3 or of Tapi-2, a pharmacological ADAM17 inhibitor, i) sensitized the cells to TNF-α-mediated hyperpermeability, ii) down-regulated tight junction-associated protein expression and iii) inhibited TNFRI shedding. In conclusion, our data showed that TACE and TIMP3 were co-expressed in the human intestinal barrier and that TACE inhibition, either physiologically or pharmacologically, amplified TNF-α-mediated hyperpermeability. TIMP3 could thus play a major role in inflammatory conditions by creating an autocrine effect leading to amplified epithelial barrier hyperpermeability.

Correspondence to: Dr Damien Masson, INSERM U539, Faculté de Médecine, 44035 Nantes, France
E-mail: masson.damien@voila.fr

Key words: inflammatory bowel disease, tissue inhibitor of metalloproteinase 3, tumor necrosis factor α, tumor necrosis factor α converting enzyme, intestinal barrier permeability

Introduction

Inflammatory bowel diseases (IBD) are characterized by defective intestinal barrier functions resulting from increased intestinal permeability (1-3), mainly due to increased tumor necrosis factor α (TNF-α) production (4-6). TNF-α is synthesized as a precursor anchored in the cytoplasmic membrane and is proteolytically cleaved at the cell surface by TACE (TNF-α converting enzyme), generating the mature secreted form of TNF-α (7,8). TACE controls both TNF-α production by inflammatory/immune cells, and the bioavailability of TNF-α receptors (TNFRs) on target cells, i.e. epithelial cells (9-11). Thus, TACE is a potential key player in the control of the intestinal barrier under physiological and pathological conditions.

The activity of TACE is regulated at three distinct levels: transcription of the gene, proenzyme activation, and through specific interaction with its physiological inhibitor tissue inhibitor of metalloproteinase 3 (TIMP3), a member of a family of endogenous matrix metalloproteinase inhibitors (12-14). TIMP3, a secreted 24-kDa protein which binds to the extracellular matrix, may be a critical regulator of the inflammatory response and a potential therapeutic protein to control inflammation through a reduction in the amount of secreted TNF-α. Thus, TACE inhibition may represent a novel approach to treat inflammation, and consequently, a large number of synthetic TACE inhibitors have been reported (15-17).

The expression and role of TIMP3 in the intestinal epithelial barrier either in normal or inflammatory conditions have not as yet been elucidated. In this study, we investigated i) the expression of TACE and TIMP3 in epithelial cells of human normal colon and inflammatory bowel diseases and ii) the effects of TACE inhibition by TIMP3 or a pharmacological inhibitor on epithelial barrier permeability in a human colonic cell line spontaneously deficient in TIMP3 (HT29-Cl.16E). We showed that TIMP3 and TACE are constitutively expressed by the human normal intestinal epithelial barrier. In inflammatory conditions, TACE inhibition by recombinant human TIMP3 or by a pharmacological agent amplifies the epithelial barrier disruption elicited by TNF-α in the TIMP3-deficient cell line HT29-Cl.16E. Our results suggest that in

IBD, TIMP3 can be an autocrine amplifier of the intestinal epithelial barrier disruption initiated by pro-inflammatory stimuli, and that TACE inhibitors can contribute to TNF-α-induced hyperpermeability of the epithelial barrier.

Materials and methods

Patients. Nineteen patients with inflammatory bowel diseases (IBD) treated at the Department of Gastroenterology (Centre Hospitalier Universitaire de Nantes, France) were included in this study. Surgical resections were obtained from 9 patients with ulcerative colitis (UC) (2 men and 7 women with a median age of 39 years; range 20-73 years) and 10 patients with Crohn's disease (CD) (1 man and 9 women with a median age of 36 years; range 21-66 years). Ulcerative colitis and Crohn's disease were diagnosed on clinical, endoscopic and histologic criteria. All patients displayed active disease, with acute or chronic inflammation. In parallel, histologically normal mucosa of patients who were treated by colectomy for colorectal carcinoma were studied as controls. All tissues were processed according to the French Guidelines for Research on Human Tissues (18).

Immunohistochemistry. Tissue microarrays of Crohn's disease and ulcerative colitis were constructed as previously described (19), and included 19 patients displaying active disease with acute or chronic inflammation. In parallel, histologically normal mucosa of colorectal cancer patients were studied. Immunohistochemistry was performed as previously described (19), using a monoclonal antibody directed against human TIMP3 (1:50, clone 136-13H4; Calbiochem Merck Biosciences, Darmstadt, Germany) or a polyclonal antibody against TACE (1:200; Chemicon, Temacula, CA, USA). A streptavidin-biotin-peroxidase method was used (LSAB Staining Kit, Dako Cytomation, Trappes, France). DAB was used as a chromogen, and sections were slightly counter-stained with hematoxylin. Controls, performed by omitting the primary antibody, were scored negative.

Isolation of epithelial cells. Human colonic epithelial cells were isolated from histologically normal colon taken at a distance (10 cm) from the tumor of surgical resections for cancer (n=15) and from inflamed colonic mucosa of surgical resections from patients with IBD (n=8). The intestinal epithelial cell isolation procedure was performed using a non-enzymatic dissociation technique, based on short-term EDTA treatment, as described previously (20). The pellets of isolated cells were lyzed in 1 ml Tri reagent (Euromedex, Mundolsheim, France), and RNA extraction was performed. Preparations of epithelial cells were devoid of contamination by lamina propria immune cells (20).

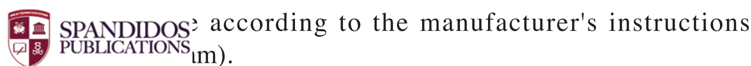
Cell culture and treatments. The experiments were performed by using a human colonic polarized cell line, HT29-Cl.16E (21,22). Cells were grown on porous filters (12-well Transwell Clear, 0.40-μm porosity; Costar) and formed monolayers of polarized cells at postconfluency. Cells were seeded at 5×10^5 cells per well in DMEM (Invitrogen, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), cultured until confluency and full differentiation, and assessed by the measurement of transepithelial resistance (TER) using a volt-ohmeter (Millipore), as well as by Alcian blue histochemistry for HT29-Cl.16E (mucus-secreting cell line) (22,23).

Experiments were conducted between day 15 and 18 after seeding. Filter-grown HT29-Cl.16E monolayers were treated with TNF-α (10 ng/ml) for 6 or 24 h. HT29-Cl.16E monolayers were incubated with 50 μM Tapi-2 (Calbiochem, San Diego, CA) or 1 μg/ml recombinant human TIMP3 (rhTIMP3, Calbiochem) for 2 h prior to stimulation with TNF-α (10 ng/ml).

Measurement of paracellular permeability. To measure changes in the paracellular permeability of HT29-Cl.16E monolayers, the cells were plated onto permeable filters in 12-well Transwell chambers (5×10^5 cells/well). Experiments were performed using confluent monolayers between day 15 and 18 after seeding. At the end of treatment with TNF-α ± Tapi-2 or rhTIMP3, the flux of fluorescein isothiocyanate-labeled dextran (molecular weight, 4 kDa; Sigma) (FITC-dextran) across HT29-Cl.16E monolayers was assayed as described previously (23). DMEM was used as the incubation solution; 1.5 ml was added to the basolateral compartment and 500 μl containing 1 mg/ml of 4-kDa FITC-dextran was added to the apical compartment. The FITC-dextran concentration was determined in the basolateral medium after 6 and 24 h of incubation at 37˚C using a fluorescent plate reader (Victor, Perkin-Elmer). All experiments were performed in triplicate.

Immunofluorescence studies. Monolayers were fixed in 4% paraformaldehyde in PBS buffer and processed for immuno-fluorescence as described previously (23,24). Cell monolayers were incubated for 1 h with a mouse anti-ZO1 or anti-occludin antibody (monoclonal, 1:200, Zymed, San Francisco, CA). After washing, cells were incubated with Alexa 488-conjugated goat anti-mouse antibody (1:200; Molecular Probes, Eugene, OR, USA). Nuclear staining was performed with Topro-3 (1 μM, Molecular Probes). Cell monolayers were then mounted using Prolong Antifade Medium (Molecular Probes). Imaging was performed on a Leica-TCS-SP confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with an argon-krypton laser. Image processing was carried out using TCS-NT software (Leica).

Immunoblot analysis. For total protein extraction, a human colonic cancer cell line (HT29-Cl.16E) was lysed in RIPA buffer supplemented with protease inhibitors and centrifuged as previously described (19). Proteins (50 μg) were separated by electrophoresis on 7.5% polyacrylamide gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Invitrogen). After blocking, membranes were incubated with a rabbit polyclonal antibody against human TACE cyto-plasmic tail (1:1000; Chemicon) or a monoclonal antibody directed against human TIMP3 (1:500, clone 136-13H4; Calbiochem Merck Biosciences), and then with an alkaline phosphatase-conjugated anti-rabbit or anti-mouse antibody (1:5000; Amersham). Immunoreactive proteins were detected with a fluorescence scanner (Storm, Pharmacia) using ECF



according to the manufacturer's instructions (...m).

RNA extraction and real-time RT-PCR analysis. RNA extraction, RT and PCR amplifications were conducted as previously described (19,24). TACE and TIMP3 mRNA were quantified using commercially available kits (Taqman Gene Expression Assays, Hs00234224 and Hs00927214, respectively; Applied Biosystems, Forster City, CA, USA), in a 7700 thermocycler using the ABI PRISM 7000 SDS software (Applied Biosystems). ZO1, occludin and ß-actin mRNA levels were quantified using the Rotorgene 2000 instrument and primer sets as described previously (23,24). Primer sets for occludin were as follows: forward, 5'ATGA GACAGACTACACAACTGG3' and reverse, 5'TTGTATTC ATCAGCAGCAGC3'. For each sample, the ratio between the relative amount of the specific transcript and ß-actin was calculated.

Analysis of CpG island methylation status. The methylation status of the TIMP3 gene promoter of the HT29-Cl.16E cells was determined by methylation-specific PCR after genomic DNA isolation using the Qiamp MiniKit (Qiagen, Valencia, CA, USA). DNA methylation patterns in the CpG islands of TIMP3 were determined by chemical conversion of unmethylated, but not methylated, cytosine to uracil and subsequent PCR using primers specific for either the methylated or modified unmethylated DNA. The primers and PCR conditions for the MS-PCR analysis were described previously (25). Each PCR reaction was directly loaded onto nondenaturing 4% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Soluble TNFRI ELISA assay. Soluble TNFRI released from cell monolayers in the basolateral incubation medium was determined by ELISA (R&D Systems), according to the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed with Statview F-4.5 (Abacus Concepts, Berkeley, CA, USA). Data are presented as the mean ± S.E. All experiments were performed at least in triplicate. The p-value was determined by the Mann-Whitney's unpaired test and was considered significant at values <0.05.

Results

TIMP3 and TACE expression in the epithelial compartment in normal mucosa and in inflammatory bowel diseases. In the human normal colon, epithelial cells expressed both TIMP3 and TACE (Fig. 1A and C). Endothelial cells and some immune cells also expressed both TIMP3 and TACE. However, muscularis mucosae and vascular myocytes expressed only TACE. TACE and TIMP3 expression was then examined in active IBD. In all the IBD samples, TIMP3 was strongly expressed by the inflammatory mononuclear cell infiltrates and by regenerative epithelial cells of the crypts (Fig. 1D). In the crypt epithelium, a strong staining intensity was observed in all cases of IBD, both in Crohn's disease and in ulcerative colitis. The same expression pattern occurred with TACE, with a strong staining intensity in crypt epithelial cells of IBD (Fig. 1B).

Quantification of mRNA levels using real-time PCR, in epithelial cells isolated from normal colon or IBD samples, showed that both TACE and TIMP3 were expressed in IBD and in normal colon. The difference was not statistically significant but there was a trend towards a higher expression in IBD samples (Fig. 1E and F). The high expression of TIMP3 in epithelial cells both in normal colon and in IBD prompted us to analyse the consequences of TACE inhibition on colonic epithelial cells *in vitro* in inflammatory conditions. We addressed this issue by using the colonic differentiated epithelial cell line, HT29-Cl.16E.

TACE and TIMP3 expression and regulation by TNF-α in a human colonic cell line, HT29-Cl.16E. We first examined the status of TACE in HT29-Cl.16E, a human colonic cancer cell line. Immunoblot analysis showed that HT29-Cl.16E cells expressed both the 115-kDa proform and the 90-kDa active form of TACE (Fig. 2A). We then analysed TACE mRNA transcripts by qRT-PCR in control and inflammatory conditions. As shown in Fig. 2B, TACE was expressed in control monolayers, and its expression was significantly higher in TNF-α-treated cells ($p=0.028$). In contrast, barely detectable levels of TIMP3 mRNA were found in these cells, both in untreated and in TNF-α-treated cells (Fig. 2B). In agreement with these results, TIMP3 was not detected in these cells by immunoblot analysis, whereas Caco-2 cells, used as positive controls, were found to express TIMP3, which was detected as a dimer (data not shown).

The TIMP3 promoter has been shown to be methylated in various tumors and cancer cell lines, including HT-29 cells (25). In order to determine whether methylation of the *TIMP3* promoter was responsible for this absence of expression, MS-PCR was performed. As shown in Fig. 2C, the *TIMP3* promoter was found to be methylated in the HT29-Cl.16E cell line.

We therefore used this cell line in further experiments as a model for studying TACE inhibition and to examine the role of exogenous recombinant human TIMP3 (rhTIMP3), mimicking the secreted TIMP3 *in vivo* on epithelial cells in inflammatory conditions.

Effect of TACE inhibition by TIMP3 or a pharmacological inhibitor on intestinal epithelial barrier function. We then assessed the possible role of TIMP3 on epithelial barrier permeability in HT29-Cl.16E monolayers treated by TNF-α. A 24-h treatment of confluent HT29-Cl.16E monolayers grown on filters with TNF-α (10 ng/ml) induced a significant increase in 4-kDa FITC-dextran flux compared with control HT29-Cl.16E monolayers (Fig. 3A). The addition of rhTIMP3 to TNF-α-treated monolayers, at a concentration (1 μg/ml) corresponding to its IC_{50} determined in *in vitro* studies (12), significantly increased the TNF-α-induced FITC-dextran flux ($p<0.0001$, Fig. 3A). TIMP3 alone did not modify FITC-dextran flux as compared with control untreated monolayers. Notably, Tapi-2, a pharmacological TACE inhibitor of the hydroxamate family, also significantly increased the TNF-α-induced FITC-dextran flux ($p=0.0079$), whereas Tapi-2 by itself did not modify the paracellular dextran flux (Fig. 3B). Altogether, these findings indicated that TACE inhibition,

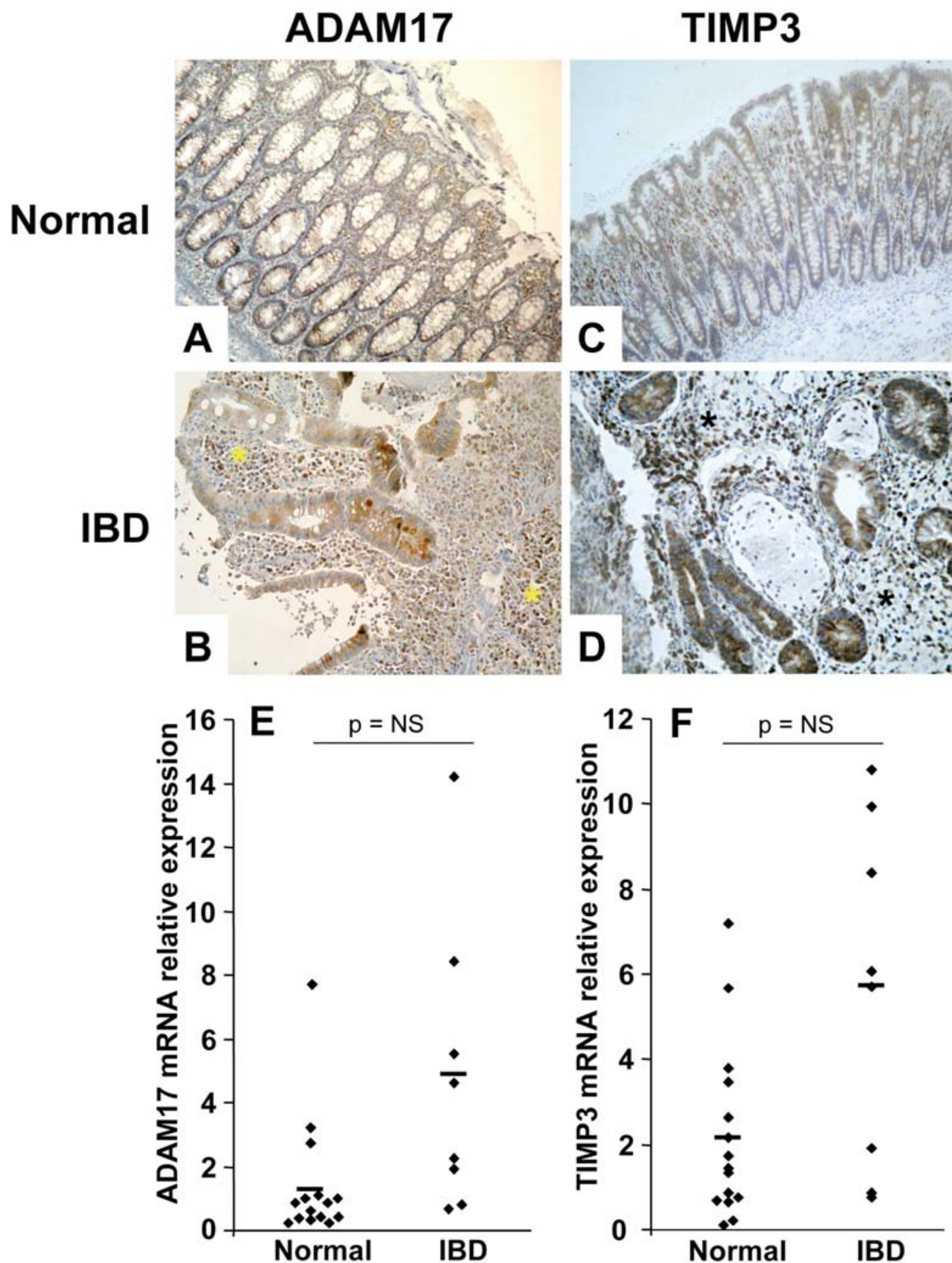


Figure 1. ADAM17 (TACE) and TIMP3 expression in the epithelial compartment both in normal mucosa and in inflammatory bowel diseases. (A and B) ADAM17 immunohistochemistry. In the normal colonic mucosa (A) ADAM17 was expressed by epithelial cells and some immune cells. This expression was high in IBD (B). The mononuclear cell infiltrates also strongly expressed ADAM17 (* in yellow). (C and D) TIMP3 immunohistochemistry. In the normal colon (C), TIMP3 was expressed in epithelial cells, as well as in some inflammatory mononuclear cells of the lamina propria. In IBD (D), the intensity of TIMP3 immunostaining was strong in regenerative epithelial cells of the crypts. Most of the mononuclear cells infiltrates were strongly immunolabelled for TIMP3 (* in black). Representative pictures are shown. (E and F) Relative expression of ADAM17 (E) and TIMP3 (F) mRNA levels in epithelial cells isolated from human normal colonic mucosa (n=15) and from IBD samples (n=8). Results are expressed relative to ß-actin levels. Lines represent mean values.

either physiologically by rhTIMP3 or pharmacologically by Tapi-2, sensitized intestinal epithelial monolayers to respond to TNF-α.

We then examined the effects of TACE inhibition on the regulation of two tight junction-associated proteins, ZO-1 and occludin, at the mRNA level using real-time PCR. TACE inhibition and TNF-α simultaneous treatment resulted in a significant decrease in ZO-1 and occludin mRNA levels compared with treatment with TNF-α alone (Fig. 4A and B).

ZO-1 expression was then examined at the protein level by immunofluorescence followed by confocal microscopy (Fig. 4C). This study showed a strong and continuous pericellular labelling in control HT29-Cl.16E monolayers. ZO-1 membrane expression was decreased and discontinuous after TNF-α treatment (10 ng/ml for 24 h). TACE inhibition together with TNF-α treatment worsened ZO-1 alterations observed with TNF-α alone: ZO-1 membrane labelling was completely disrupted, faint in some areas, sometimes

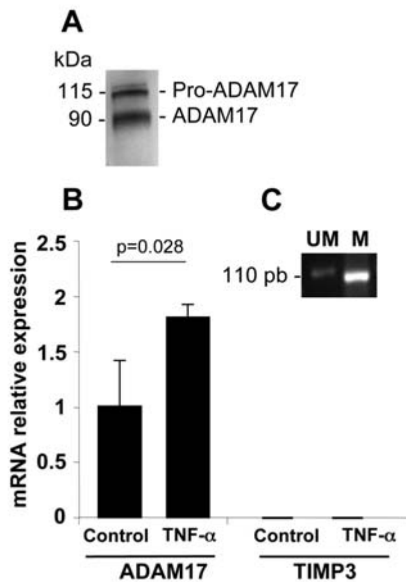


Figure 2. ADAM17 (TACE) and TIMP3 expression in the human colonic cell line HT29-Cl.16E. (A) Immunoblot analysis of ADAM17. HT29-Cl.16E cells expressed the proform and active forms of ADAM17. Molecular mass is shown on the left. (B) ADAM 17 and TIMP3 mRNA expression under inflammatory stress was determined by real-time PCR in the HT29-Cl.16E cell line. The ADAM17 mRNA level was significantly increased (p=0.028) by TNF-α (10 ng/ml, 24 h) compared with the controls, while TIMP3 mRNA was not expressed in either the control or TNF-α treated cells. Mean ± S.E. of 6 monolayers. (C) Methylation pattern of *TIMP3* promoter in HT29-Cl.16E cells by MS-PCR. HT29-Cl.16E cells exhibited a methylated (M) pattern of the *TIMP3* promoter.

reinforced at cellular contact spots, and ZO-1 was redistributed to the cytoplasm in some cells. Tapi-2 alone did not modify ZO-1 labelling as compared with the controls. Similarly to ZO-1, occludin protein expression was decreased and disorganized in TNF-α- and TNF-α + Tapi-2-treated monolayers (data not shown).

Effect of TACE inhibition on TNFRI shedding by the HT29-Cl.16E cells. These results suggested that TACE inhibition stabilized the TNF-α receptors type I (TNFRI) at the surface of epithelial cells and thus sensitized epithelial cells to TNF-α. To test this hypothesis, we measured by ELISA the release of TNFRI in the culture media of HT29-Cl.16E monolayers treated by TNF-α, with or without inhibition of TACE. As shown in Fig. 5, TACE inhibition alone or in association with TNF-α strongly decreased TNFRI release in the supernatant.

Discussion

Our research demonstrated that TACE and TIMP3 are co-expressed in the epithelial intestinal barrier and that TIMP3 synergizes the deleterious effect of TNF-α on the intestinal epithelial barrier function through TACE inhibition; these effects being mimicked by a pharmacological TACE inhibitor.

Several lines of investigation, mainly based on *timp3*$^{-/-}$ mice or on gene transfer experiments *in vitro*, have shown that TIMP3 is able to inhibit an inflammatory response (26). They showed that TIMP3 is essential for normal innate immune function and that the loss of TIMP3 impacts innate immunity by dysregulating cleavage of TNF-α and its receptors. Furthermore, they postulated that TIMP3-based therapies can be developed to control inflammation. Indeed, *timp3*$^{-/-}$ mice that have a normal life, but exhibit an inflammatory phenotype, are more susceptible to LPS-induced mortality and show an increased inflammatory response to intra-articular antigen injection. Thus, it was concluded that TIMP3 is a critical regulator of the inflammatory response (26-28). In the current study, we examined the status and the role of TIMP3 in IBD. We focused on the intestinal epithelial compartment which expresses TNFRs and is thus a possible target of TNF-α. In this cellular compartment, TIMP3, known to regulate the availability of TNFRs on the cell surface through TACE inhibition (9), may play an important role. Up to now, TACE expression has been reported in the epithelial compartment (19,29,30) but only one study reported TIMP3 expression in the normal and inflammatory intestinal barrier, although data was not shown (31). In our study, immuno-

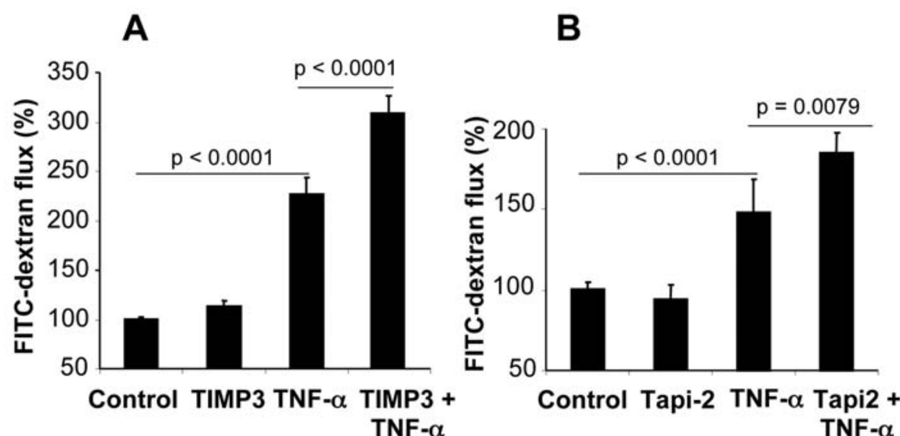


Figure 3. Effect of ADAM17 (TACE) inhibition on HT29-Cl.16E paracellular permeability. (A and B) Paracellular flux of 4-kDa FITC-dextran was measured in confluent HT29-Cl.16E monolayers treated or not with TNF-α (10 ng/ml, 24 h) in combination with rhTIMP3 (1 μg/ml) (A) or the pharmacological ADAM17 inhibitor Tapi-2 (50 μM) (B). Results are expressed as percent increase of control untreated monolayers. rhTIMP3 and Tapi-2 significantly increased the TNF-α-induced FITC-dextran flux. Mean ± S.E. of 6 monolayers.

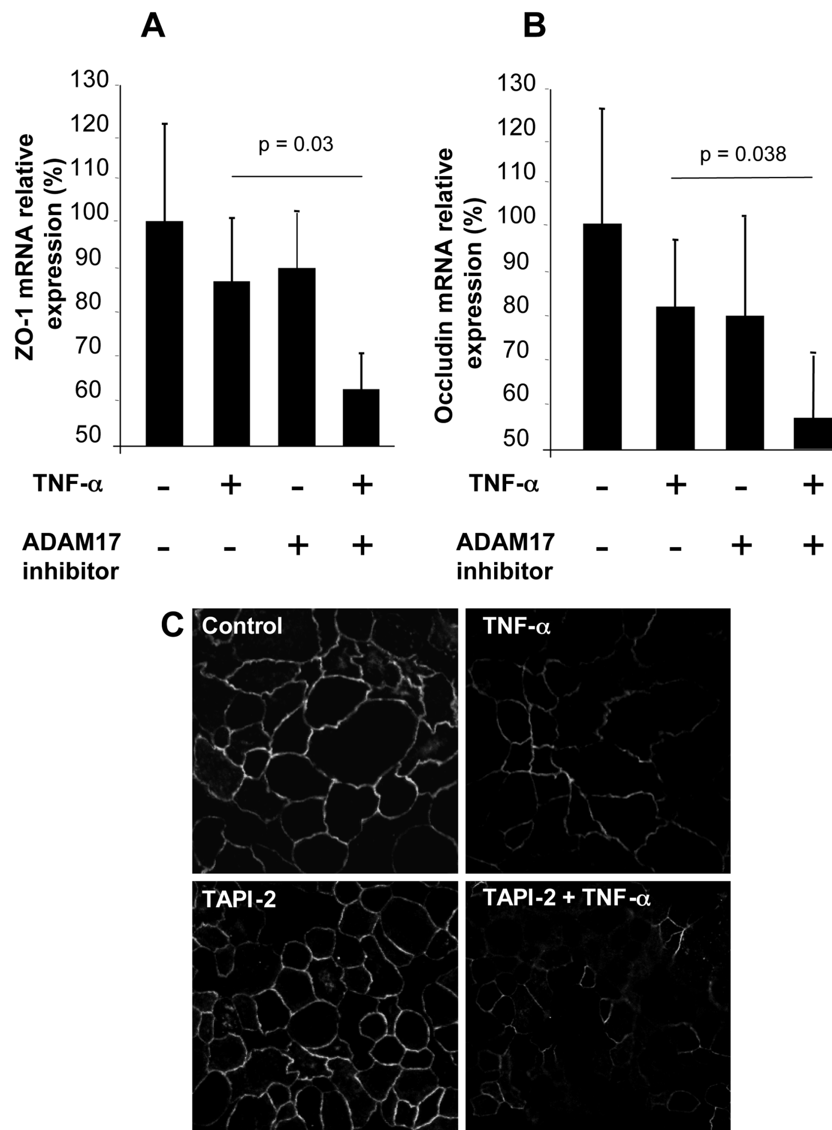


Figure 4. Effect of ADAM17 (TACE) inhibition on HT29-Cl.16E tight junction-associated protein expression. ZO-1 (A) and occludin (B) mRNA levels were determined by real-time PCR as described in Materials and methods. Results are expressed considering a value of 100% for untreated control cells. ADAM17 inhibition and TNF-α simultaneous treatment resulted in a significant decrease in ZO-1 and occludin mRNA levels compared with treatment with TNF-α alone. Mean ± S.E. of 6 monolayers. (C) Immunofluorescence staining followed by confocal microscopic examination showing a decreased and disorganized ZO-1 protein expression in TNF-α- or TNF-α + Tapi-2-treated HT29-Cl.16E monolayers compared with control monolayers. Representative images are shown.

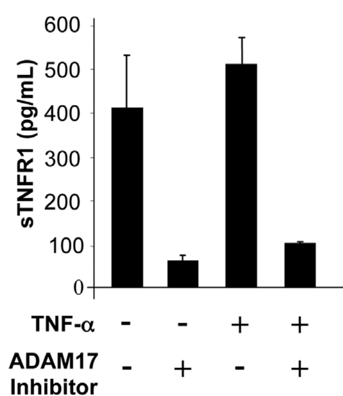


Figure 5. Effect of ADAM17 (TACE) inhibition on TNFRI shedding by HT29-Cl.16E cells. Soluble TNFRI was measured by ELISA in culture media of HT29-Cl.16E cells after basolateral exposure to TNF-α (10 ng/ml, 24 h), ADAM17 inhibitor (Tapi-2, 50 μM) or both. Tapi-2 induced a significant inhibition in TNFRI shedding. Mean ± S.E. of 7 monolayers from 2 experiments.

histochemical analysis showed that both TACE and TIMP3 are expressed by human intestinal epithelial cells, in normal colon and in IBD patients, putting forward the concept of an autocrine regulatory role of TIMP3 on TACE function in intestinal epithelial cells. In addition, TACE and TIMP3 expression was confirmed at the mRNA level in isolated epithelial cells from IBD patients and normal colon.

To get insight into the role of TIMP3 in the intestinal epithelium in the context of the inflammatory process, we then used a mechanistic approach. The human colonic epithelial cell line HT29-Cl.16E was maintained on porous filters as polarized and differentiated monolayers. HT29-Cl.16E expressed the active form of TACE but showed a methylation pattern, which was responsible for the lack of TIMP3 mRNA expression and was resistant to TIMP3 induction by TNF-α. We then examined the role of exogenous TIMP3 in combination with TNF-α in the TIMP3-deficient HT29-Cl.16E monolayers. The epithelial barrier function was

d by assessing i) paracellular permeability using the test of FITC-dextran flux across monolayers and ii) the regulation of two tight junction-associated proteins playing a key role in epithelial homeostasis: ZO-1 and occludin.

One major finding of this study was the potentiation of the TNF-α-increased paracellular permeability and tight junction protein alteration by TIMP3. In the HT29-Cl.16E cell line, we showed that rhTIMP3 alone did not alter paracellular permeability. However, in TNF-α-treated monolayers, TIMP3 increased TNF-α-mediated hyperpermeability. Notably, these effects of rhTIMP3 were reproduced with a pharmacological inhibition of TACE, Tapi-2, a member of the hydroxamate family. Hydroxamates are increasingly being developed as potential pharmaceutical agents, and a series of orally bioavailable selective TACE inhibitors are currently in development (32). As TNF-α is known to cause intestinal epithelial barrier disruption by apoptosis-dependent and -independent mechanisms via down-regulation of tight junction-associated proteins (33-36), we verified that TACE inhibition-mediated potentiation of TNF-α-mediated hyperpermeability was associated with down-regulation of ZO-1 and occludin.

The process of TNFR shedding by TACE is important, as it up-regulates soluble receptor production and also provides a mechanism for desensitizing cells to the biological effects of the ligand TNF-α. This is achieved by reducing the concentration of surface receptors, thus modulating the signalling and also by inhibiting the ligand competitively. It can be postulated that inhibition of TNFR shedding by TIMP3 or a pharmacological inhibitor may account for the amplification of TNF-α deleterious effects on target cells (37). This concept was supported in our study by a major reduction in TNFRI shedding by a pharmacological inhibitor of TACE, which also validated that TACE was functionally bioactive in our experiments.

TIMP3-based therapies can be considered as novel approaches to control inflammation (16), in the context of the emergence of new agents able to block TNF-α (38,39). Thus, as no totally specific TACE inhibitor is available yet, the identification of highly selective TACE inhibitors is a major goal for pharmaceutical industries. However, our findings suggest that the pharmacological blockade of TACE might have adverse effects in the intestine and may locally worsen epithelial barrier disruption in an inflammatory context. Similar findings have been described in experimental arthritis, where TACE inhibition was associated with increased inflammation (40). This could be linked to the inhibition of metalloproteases other than TACE and with the modulation of TNFR shedding (41).

Altogether, our data show that TIMP3 can be considered as a critical regulator of the inflammatory response by controlling the bioavailability of the TNF-α receptors on intestinal epithelial cells. It could thus play a major role in inflammatory conditions, by creating an autocrine effect leading to amplified epithelial barrier hyperpermeability.

Acknowledgements

This study was supported in part by a grant from Schering Plough/SNFGE (BREMICI). The authors thank Mrs. M.A. Martinez for the critical reading and helpful discussion.

References

1. Clayburgh DR, Shen L and Turner JR: A porous defense: the leaky epithelial barrier in intestinal disease. Lab Invest 84: 282-291, 2004.
2. Sanders DS: Mucosal integrity and barrier function in the pathogenesis of early lesions in Crohn's disease. J Clin Pathol 58: 568-572, 2005.
3. Sartor RB: Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 3: 390-407, 2006.
4. Van Deventer SJ: Tumour necrosis factor and Crohn's disease. Gut 40: 443-448, 1997.
5. Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA and Said HM: TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. Am J Physiol Gastrointest Liver Physiol 286: 367-376, 2004.
6. Turner JR: Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. Am J Pathol 169: 1901-1909, 2006.
7. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, Leber TM, Mangan M, Miller K, Nayee P, Owen K, Patel S, Thomas W, Wells G, Wood LM and Woolley K: Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 370: 555-557, 1994.
8. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ and Cerretti DP: A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 385: 729-733, 1997.
9. Smith MR, Kung H, Durum SK, Colburn NH and Sun Y: TIMP-3 induces cell death by stabilizing TNF-alpha receptors on the surface of human colon carcinoma cells. Cytokine 9: 770-780, 1997.
10. Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ and Black RA: An essential role for ectodomain shedding in mammalian development. Science 282: 1281-1284, 1998.
11. Schlondorff J and Blobel CP: Metalloprotease-disintegrins: modular proteins capable of promoting cell-cell interactions and triggering signals by protein-ectodomain shedding. J Cell Sci 112: 3603-3617, 1999.
12. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, Stephens PE, Shelley C, Hutton M, Knauper V, Docherty AJ and Murphy G: TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435: 39-44, 1998.
13. Schlondorff J, Becherer JD and Blobel CP: Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). Biochem J 347: 131-138, 2000.
14. Peiretti F, Canault M, Deprez-Beauclair P, Berthet V, Bonardo B, Juhan-Vague I and Nalbone G: Intracellular maturation and transport of tumor necrosis factor alpha converting enzyme. Exp Cell Res 285: 278-285, 2003.
15. Holms J, Mast K, Marcotte P, Elmore I, Li J, Pease L, Glaser K, Morgan D, Michaelides M and Davidsen S: Discovery of selective hydroxamic acid inhibitors of tumor necrosis factor-alpha converting enzyme. Bioorg Med Chem Lett 11: 2907-2910, 2001.
16. Newton RC, Solomon KA, Covington MB, Decicco CP, Haley PJ, Friedman SM and Vaddi K: Biology of TACE inhibition. Ann Rheum Dis 60: 25-32, 2001.
17. Beck G, Bottomley G, Bradshaw D, Brewster M, Broadhurst M, Devos R, Hill C, Johnson W, Kim HJ, Kirtland S, Kneer J, Lad N, Mackenzie R, Martin R, Nixon J, Price G, Rodwell A, Rose F, Tang JP, Walter DS, Wilson K and Worth E: (E)-2(R)-[1(S)-(hydroxycarbamoyl)-4-phenyl-3-butenyl]-2'-isobutyl-2'-(methanesulfonyl)-4 methylvalerohydrazide (Ro 32-7315), a selective and orally active inhibitor of tumor necrosis factor-alpha convertase. J Pharmacol Exp Ther 302: 390-396, 2002.
18. Agence Nationale d'Accreditation et d'Evaluation en Sante (ANAES): Recommendations for cryopreservation of cells tumor tissues to be used for molecular analyses. Ann Pathol 21: 184-201, 2001.
19. Blanchot-Jossic F, Jarry A, Masson D, Bach-Ngohou K, Paineau J, Denis MG, Laboisse CL and Mosnier JF: Up-regulated expression of ADAM17 in human colon carcinoma: co-expression with EGFR in neoplastic and endothelial cells. J Pathol 207: 156-163, 2005.

20. Branka JE, Vallette G, Jarry A, Bou-Hanna C, Lemarre P, Van PN and Laboisse CL: Early functional effects of *Clostridium difficile* toxin A on human colonocytes. Gastroenterology 112: 1887-1894, 1997.
21. Fogh J, Fogh JM and Orfeo T: One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J Natl Cancer Inst 59: 221-226, 1977.
22. Augeron C and Laboisse CL: Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Res 47: 3961-3969, 1984.
23. Neunlist M, Toumi F, Oreschkova T, Denis M, Leborgne J, Laboisse CL, Galmiche JP and Jarry A: Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. Am J Physiol 285: G1028-G1036, 2003.
24. Jarry A, Bach-Ngohou K, Masson D, Dejoie T, Lehur PA, Mosnier JF, Denis MG and Laboisse CL: Human colonic myocytes are involved in postischemic inflammation through ADAM17-dependent TNFalpha production. Br J Pharmacol 147: 64-72, 2006.
25. Bachman KE, Herman JG, Corn PG, Merlo A, Costello JF, Cavenee WK, Baylin SB and Graff JR: Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggests a suppressor role in kidney, brain, and other human cancers. Cancer Res 59: 798-802, 1999.
26. Smookler DS, Mohammed FF, Kassiri Z, Duncan GS, Mak TW and Khokha R: Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation. J Immunol 176: 721-725, 2006.
27. Mohammed FF, Smookler DS, Taylor SE, Fingleton B, Kassiri Z, Sanchez OH, English JL, Matrisian LM, Au B, Yeh WC and Khokha R: Abnormal TNF activity in Timp3$^{-/-}$ mice leads to chronic hepatic inflammation and failure of liver regeneration. Nat Genet 36: 969-977, 2004.
28. Mahmoodi M, Sahebjam S, Smookler D, Khokha R and Mort JS: Lack of tissue inhibitor of metalloproteinases-3 results in an enhanced inflammatory response in antigen-induced arthritis. Am J Pathol 166: 1733-1740, 2005.
29. Brynskov J, Foegh P, Pedersen G, Ellervik C, Kirkegaard T, Bingham A and Saermark T: Tumour necrosis factor alpha converting enzyme (TACE) activity in the colonic mucosa of patients with inflammatory bowel disease. Gut 51: 37-43, 2002.
30. Kirkegaard T, Pedersen G, Saermark T and Brynskov J: Tumour necrosis factor-alpha converting enzyme (TACE) activity in human colonic epithelial cells. Clin Exp Immunol 135: 146-153, 2004.
31. Kirkegaard T, Hansen A, Bruun E and Brynskov J: Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. Gut 53: 701-709, 2004.
32. Levin JI, Chen JM, Laakso LM, Du M, Schmid J, Xu W, Cummons T, Xu J, Jin G, Barone D and Skotnicki JS: Acetylenic TACE inhibitors. Part 3: Thiomorpholine sulfonamide hydroxamates. Bioorg Med Chem Lett 16: 1605-1609, 2006.
33. Schmitz H, Fromm M, Bentzel CJ, Scholz P, Detjen K, Mankertz J, Bode H, Epple HJ, Riecken EO and Schulzke JD: Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6. J Cell Sci 112: 137-146, 1999.
34. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM and Nusrat A: Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol 171: 6164-6172, 2003.
35. Ma TY, Boivin MA, Ye D, Pedram A and Said HM: Mechanism of TNF-α modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol 288: G422-G430, 2005.
36. Wang WV, Graham Y, Wang ED, Witkowski BT, Schwarz JR and Turner JR: Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol 166: 409-419, 2005.
37. Wang F, Schwarz BT, Graham WV, Wang Y, Su L, Clayburgh DR, Abraham C and Turner JR: IFN-gamma-induced TNFR2 expression is required for TNF-dependent intestinal epithelial barrier dysfunction. Gastroenterology 131: 1153-1163, 2006.
38. Sandborn WJ, Feagan BG, Hanaeur SB, Present DH, Sutherland LR, Kamm MA, Wolf DC, Baker JP, Hawkey C, Archambault A, Bernstein CN, Novak C, Heath PK and Targan SR: CDP571 Crohn's Disease Study Group: An engineered human antibody to TNF (CDP571) for active Crohn's disease: a randomized double-blind placebo-controlled trial. Gastroenterology 120: 1330-1338, 2001.
39. Van Deventer SJ: New biological therapies in inflammatory bowel disease. Best Pract Res Clin Gastroenterol 17: 119-130, 2003.
40. Williams LM, Gibbons DL, Gearing A, Maini RN, Feldmann M and Brennan FM: Paradoxical effects of a synthetic metallo-proteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF alpha processing in RA synovial membrane cell cultures. J Clin Invest 97: 2833-2841, 1996.
41. Van Deventer SJ: A place for TACE. Gut 51: 5-6, 2002.