TIMP-1 inhibition of occludin degradation in Caco-2 intestinal cells: a potential protective role in necrotizing enterocolitis

Amir Bein[1], Ronit Lubetzky[2,3], Dror Mandel[2,3] and Betty Schwartz[1]

BACKGROUND: Necrotizing enterocolitis (NEC), a common intestinal disease affecting premature infants, is a major cause of morbidity and mortality. Previous reports indicate an upregulation of intestinal matrix metalloproteinases (MMPs) activity that may play key roles on the higher permeability of the intestinal barrier, typical to NEC. Recently, TIMP-1, a natural inhibitor of MMP's, was found to be over expressed in preterm human breast milk (HBM). Previous studies have shown that infants fed with HBM have a significant reduction in the incidence of NEC. The aim of the present study was to investigate the possible role that TIMP-1 may play on the maintenance of tight junctions and therefore the gut barrier integrity.

METHODS: Timp-1-treated Caco-2 intestinal cells were tested for MMP-2 enzymatic activity and cell junction integrity.

RESULTS: TIMP-1 inhibited MMP-2 activity, which induced a significant increase in the expression of occludin but not of claudin-4. TIMP-1 did not affect apoptosis.

CONCLUSION: One of the putative mechanisms associated with HBM protection against NEC is mediated by TIMP-1, which downregulates MMP-2 activity, inhibits the degradation of occluding, and preserves tight junctions and gut barrier integrity.

Necrotizing enterocolitis (NEC) is the most common and severe intestinal inflammatory disease occurring principally in very-low-birth-weight premature infants (1–3). Infants able to survive NEC are susceptible to complications such as impaired nervous system and/or development of short bowel syndrome (2,4). Even though NEC represents a significant health threat and a common topic for research for quite a while, the current understanding of the pathological mechanisms associated with the development of this devastating disease is still very scant.

A striking fact is that infants fed with human breast milk (HBM) have a significant reduced incidence of NEC, reaching a ratio of up to 1:10 when compared to infants fed milk formula (5–8). The reason why HBM exerts such a protective effect finally avoiding NEC development remains to be determined.

It is well known that one of the most important anatomical and physiological features of the gut barrier is the tight junctions. Additionally, it is well established that the gut barrier in NEC patients is severely damaged (9). The tight junctions are large protein complexes situated at the apical paracellular junction of epithelial cells. They are considered as the "gate keepers" maintaining the epithelial-barrier of the intestine impermeable (10). One of the key tight junction proteins is occludin which is a ~65-kDa tetra-span protein with two extracellular loops. An additional member is the claudin family of proteins consisting of at least 24 variants ranging from 20 to 27 kDa. Even though claudins do not share sequence similarity with occludin, they comprise also a tetra-span transmembrane protein with two extracellular loops (10).

Matrix metalloproteinases (MMPs) are a group of endopeptidases that play a key role in the degradation of extracellular matrix (11–15). The natural inhibitors of MMPs are the tissue inhibitors of metalloproteinases (TIMPs), which are produced by the same cell types that produce MMPs and regulate their proteolytic function (16). Some studies have demonstrated that several MMPs are upregulated in NEC (17). MMP-1 was previously detected in NEC samples in fibroblast-like cells of the stroma and in epithelial cells of regenerating areas (18). Bister *et al.* (18) demonstrated that several other MMPs in addition to MMP-1 may play key roles on tissue destruction and remodeling in NEC. It has been also previously shown that MMP-3 and TIMP-1 transcripts were upregulated in NEC compared with control samples, whereas MMP-1, MMP-2, MMP-9, and TIMP-2 transcripts remained unaltered (15). We have recently found that MMP-2 and MMP-9 are expressed in HBM (11) and thus suggested that balance between extracellular proteases and their inhibitors in human preterm milk might contribute to the beneficial biological effects of breast milk upon the premature gut.

The characteristic cleavage activity of MMP-2 (also known as gelatinase A), is breakdown of peptide bonds located in areas rich in amino acids containing an hydrophobic side chains such as phenylalanine, leucine, isoleucine, and tyrosine (16). Interestingly, the amino acid sequence of occludin exhibits high prevalence of tyrosine, this is evident mainly in the first extracellular loop which contains a high number of tyrosine

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and glycine residues (~60%) (19); therefore, it is reasonable to speculate that occludin may provide a legitimate substrate for MMP-2 enzymatic activity.

Changes in the balance between MMP's and TIMP's levels have been found to be key determinants associated with the outcome of several pathological processes (16).

The aim of the present study was to investigate the putative role that TIMP-1 may play on preserving the tight junctions and thus the gut barrier integrity through the control of the degradation activity exerted by MMP-2. Since one of the key characteristics of NEC is degradation leading to the destruction of gut barrier, our aim was to demonstrate whether TIMP-1, a molecule found in HBM, exerts a protective effect of NEC development through the specific inhibition of MMP-2 enzymatic activity.

RESULTS

Caco-2 Cells Secrete MMP-2

Figure 1 exemplifies a zymography conducted on 10% SDS-PAGE, containing 0.1% (w/v) gelatin used to test the expression and activity of MMP-2 present in conditioned media (CM) harvested from cultured Caco-2 cells. CM from cells cultured in medium supplemented with 20% (v/v) fetal bovine serum (FBS) showed a band slightly above 70 kDa molecular weight marker, corresponding to the 72 kDa proenzyme form of MMP-2. In contrast, CM harvested from cells cultured in

Figure 1. Gelatin zymography indicating matrix metalloproteinase-2 (MMP-2) activity. MMP-2 in its proenzyme form (~72 kDa) is secreted into the conditioned media (CM) of Caco-2 cells cultured in medium supplemented with 20% fetal bovine serum (FBS) (control), whereas Caco-2 cells cultured in medium supplemented with 0.5% FBS secrete into their CM the active form of MMP-2 (~64 kDa).

medium supplemented with 0.5% (v/v) FBS showed a band at ~64 kDa, corresponding to the active form of MMP-2.

Inhibition of MMP-2 By the Recombinant TIMP-1 Molecule

To test the ability of TIMP-1 to inhibit MMP-2 endopeptic activity, different concentrations of recombinant TIMP-1 (10, 100, and 200 ng/ml), were tested while the known inhibitor—NNGH provided the control activity. **Figure 2a** shows a representative graph describing the kinetics of MMP-2 inhibition. The calculated remaining activity of MMP-2 is shown in **Figure 2b**. While TIMP-1 at a concentration of 10 ng/ml exerted only a minor inhibitory effect on MMP-2 activity (7%), higher concentrations of TIMP-1 (100 and 200 ng/ml), showed a significant inhibitory effect in a dose-dependent manner. TIMP-1 at a concentration of 100 ng/ml caused a 23% inhibition in MMP-2 activity ($P < 0.001$) while a concentration of 200 ng/ml caused a 43% inhibition in MMP-2 activity ($P < 0.0001$).

Effects of Recombinant TIMP-1 on Caco-2 Intestinal Cells Activities

TIMP-1 may interact directly with epithelial cells and affect gene expression or apoptosis (20). To assess whether TIMP-1 exerts an effect on apoptosis of Caco-2 cells, we checked for changes in the expression of apoptosis related genes. **Supplementary Figure S1** online shows that TIMP-1 did not modify the expression of the apoptosis related genes BAX and Bcl-2, a finding indicating that TIMP-1 does not affect apoptosis of treated epithelial intestinal cells.

Claudin-4 is a member of the Claudin's tight junction protein family amply expressed in the intestine. We tested whether TIMP-1 treatment has an effect on the expression of claudin-4 in Caco-2 cells. We used western blot analysis to monitor claudin-4 expression at the protein level. **Figure 3** demonstrates that the expression of Claudin-4 was not altered by any of the different concentrations of TIMP-1 used to treat Caco-2 intestinal cells.
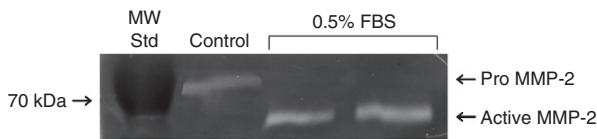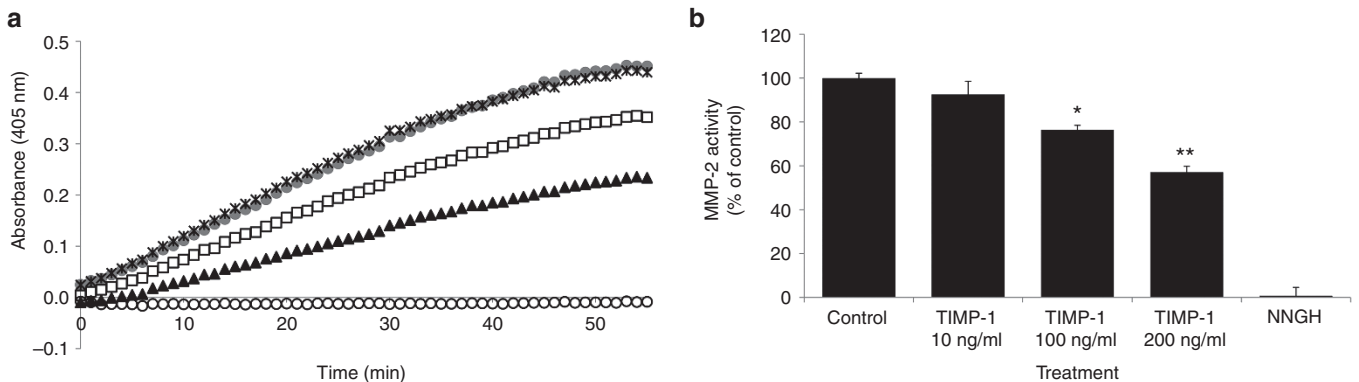

Figure 2. Effect of recombinant TIMP-1 on matrix metalloproteinase-2 (MMP-2) enzymatic activity. The thiopeptide (Ac-PLG-(2-mercapto- 4-methyl-pentanoyl)-LG-OC2H5) was used as a chromogenic substrate to monitor MMP-2 catalytic activity. Different concentrations of recombinant TIMP-1 (10, 100, and 200 ng/ml), were compared to the customary inhibitor NNGH (N-Hydroxy-2-(((4-methoxyphenyl)sulfonyl)(2-methylpropyl)amino)acetamide). (**a**) The cleavage kinetics of the chromogenic substrate by MMP-2, are represented by successive absorbance measurements at 405 nm at 1 min intervals taken for 1 h. A representative graph is shown. Control gray circles, TIMP-1 10 ng/ml asterisks, TIMP-1 100 ng/ml open squares, TIMP-1 200 ng/ml solid triangles, NNGH open circles. (**b**) The remaining activity of MMP-2 after TIMP-1 or NNGH inhibition was calculated by the slope of the kinetic graph and represented as a percentage of control. *$P < 0.0012$; **$P < 0.0001$. Data are expressed as mean ± SEM ($n = 4$).
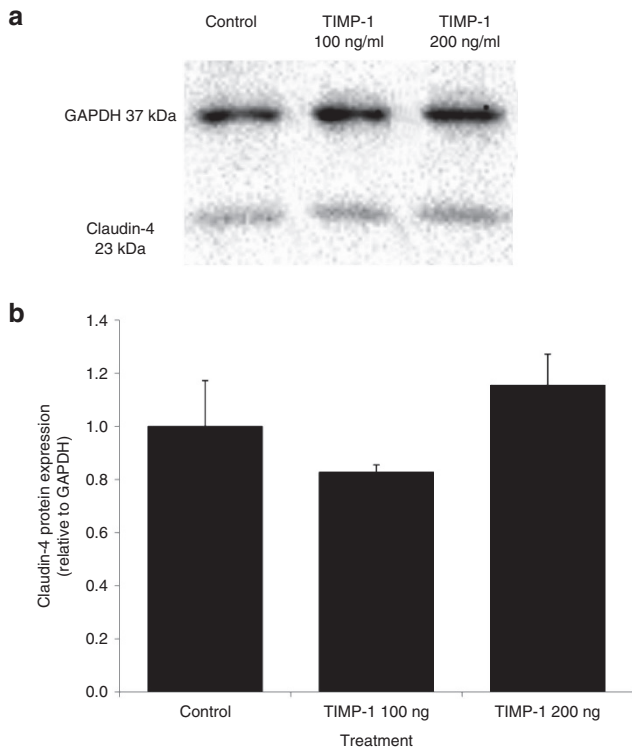
Figure 3. Effect of recombinant TIMP-1 on claudin-4 protein expression in Caco-2 cells. (**a**) Lysates of control or TIMP-1 (100 and 200 ng/ml) treated cells were used for western blot analysis. (**b**) Quantification of bands was carried out with Image Lab software (Biorad), and is represented as relative expression of claudin-4 to GAPDH. No significant differences were found. Data are expressed as mean ± SEM ($n = 9$).

We assessed herein whether different concentrations of TIMP-1 affect the expression of the genes coding for occludin, claudin-4, β-catenin, and zonula occludens 1 (ZO-1). **Figure 4** shows that TIMP-1 did not exert any significant effect on the expression of these genes coding for tight and adherence junction proteins in Caco-2 cells.

Occludin is a major tight junction protein that plays an important role in keeping the intestinal barrier impermeable (19). **Figure 5a,b**, demonstrates that TIMP-1 at 10 ng/ml, led to a moderate but not significant increase in the expression of occludin. However, in Caco-2 cells treated with 100 ng/ml of TIMP-1, a significant ($P < 0.001$) increase in the expression of occludin (more than two fold as compared to control was found). This finding was further validated by immunofluorescence staining. **Figure 5c** shows increased expression of fluorescent stained occludin (in a dose-dependent manner) as compared to control in TIMP-1-treated cells, supporting the western blot data.

DISCUSSION

Numerous studies conducted in the last decades have shed light on putative pathways involved in the development of NEC, yet we still lack an in-depth understanding of the pathophysiology of this devastating disease. In light of these limitations, the current approach to deal with this threat is treating the symptoms as they appear and trying different approaches for prevention

such as providing probiotics or feeding the preterm infant with HBM (21). While some studies have been performed in the field of probiotics and their effect on NEC (1,22), fewer studies have been conducted on the effect of HBM on NEC and even less is known about the mechanisms by which HBM components can contribute to the protective effect of HBM on NEC.

In the current study, we focused on one specific molecule found in HBM; i.e. TIMP-1, and investigated whether it plays any role in keeping the integrity of the gut barrier. Our primary assumption was that since TIMP-1 expression in HBM obtained from mothers feeding preterm infants is significantly higher than TIMP-1 expression in HBM obtained from mothers feeding term infants, it may play a role in the prevention of the proteolysis of extra cellular proteins, in particular those proteins that form the tight junctions and prevent the characteristic gut barrier damage observed in NEC patients.

The *in vitro* model system used was the Caco-2 intestinal cell line since it is of human intestinal origin and has the ability to create an epithelial monolayer with intact tight junctions. We were able to demonstrate that Caco-2 cells grown in medium supplemented with low levels of FBS (0.5%) secrete MMP-2 in its active form, whereas cells grown in medium with high levels of FBS (20%) secrete MMP-2 in its proenzyme inactive form. This model may reflect some of the characteristics of the intestinal epithelium during NEC, i.e., under stressed conditions (nutrient restriction) the cells secrete active MMP-2, whereas nonstressed cells secrete inactive MMP-2 (see **Figure 1**). We concentrated on MMP-2 since we speculate that this metalloproteinase may play a constitutive physiological role in the healthy intestinal epithelium and in contrast it may play a destructive role during NEC development. We base this assumption on the fact that it was previously reported that in NEC MMP's activities are out of balance (15) and that MMP-2 was found to be highly expressed in HBM (11).

Almost all MMPs can be inhibited by all four TIMPs, but differences in binding affinity have been reported (23,24). In the present study, the ability of TIMP-1 to inhibit MMP-2 activity was analyzed. Our results show that TIMP-1 inhibits MMP-2 activity in a dose-dependent manner with a significant 23% ($P < 0.0012$) inhibition at 100 ng/ml and up to 43% ($P < 0.0001$) at 200 ng/ml. We chose to focus our study on a TIMP-1 concentration of 100 ng/ml, since it is the minimal effective protein level able to induce a significant inhibition of MMP-2 activity.

Previous studies (20,25) demonstrate that TIMP-1 is able to translocate into the nucleus and exert a potential role in regulation of the expression of several genes. We therefore evaluated the putative effect of TIMP-1 on apoptosis which is an important process affecting the epithelial monolayer integrity. Evaluation of apoptosis was monitored based on the expression of two key genes involved in this process, i.e., Bax and Bcl-2 (26). We found that TIMP-1 at concentrations of 10 and 100 ng/ml did not induce any effect on the expression of the gene coding to the proapoptotic protein Bax nor on the expression of the gene coding to the antiapoptotic protein Bcl-2 in Caco-2 intestinal cells.
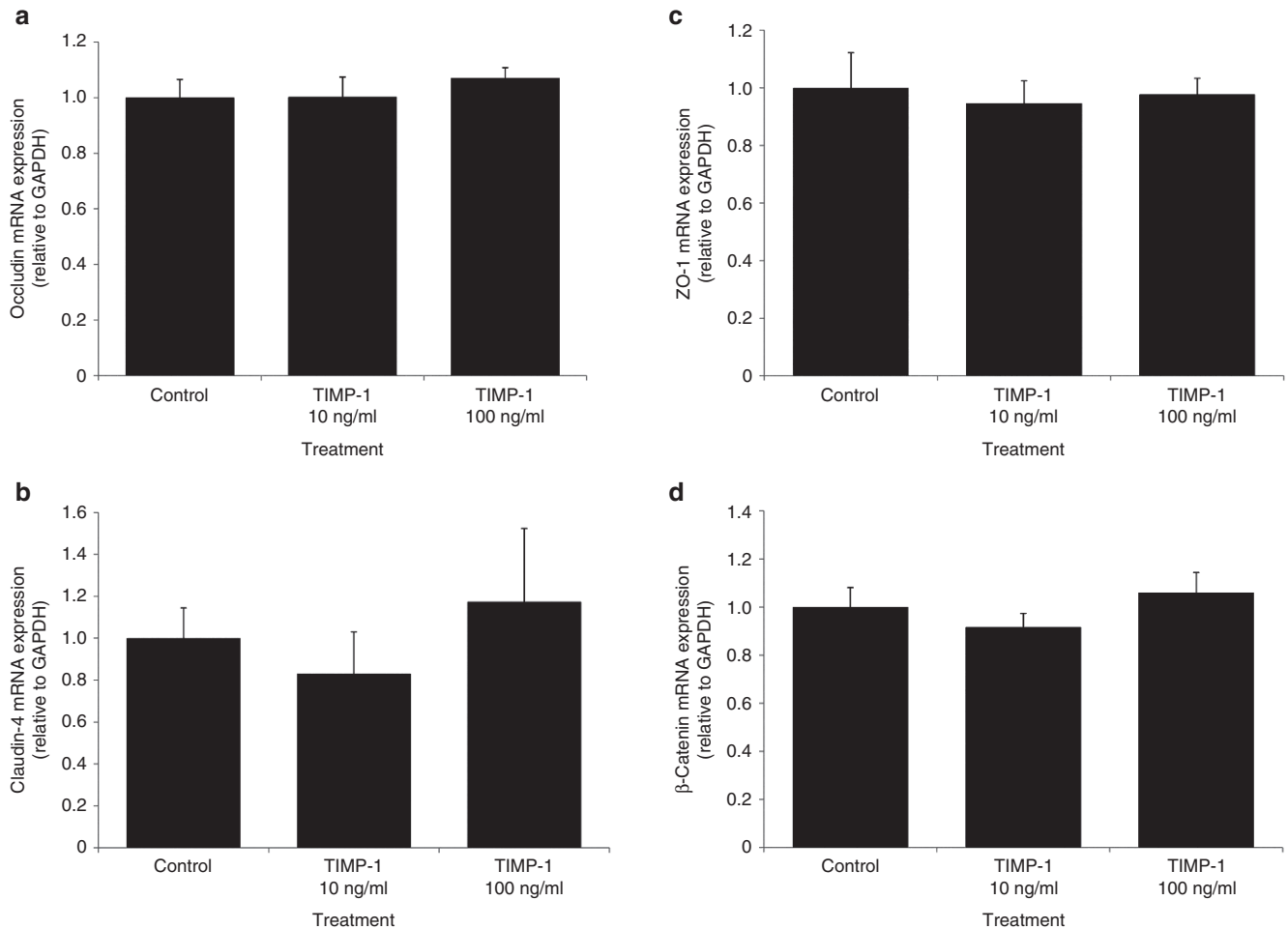
Figure 4. Effect of recombinant TIMP-1 on tight and adherence junctions' gene expression in Caco-2 cells. (**a–d**) Transcripts expression of tight junction proteins- occludin, claudin-4, ZO-1 and adherence junction protein- β-catenin, were evaluated via real time PCR analysis. Total mRNA from control cells or cells treated with TIMP-1 (10 and 100 ng/ml) was converted to cDNA and loaded with specific primers for the real time PCR reactions. No significant differences were found in all genes. Results are presented as relative expression to GAPDH. Data are expressed as mean ± SEM ($n = 19$).

Since one of the characteristics of NEC pathology is the disarray that the gut barrier experiences, the main goal of our study was therefore, to investigate the effect of TIMP-1 on the expression of specific tight junction proteins. Based on findings from previous studies that TIMP-1 is able to translocate into the nucleus and play an unidentified role in nuclear function, such as control of gene expression (20,25) we firstly assessed whether TIMP-1 affects transcription of genes coding for tight and adherence junction proteins. TIMP-1did not alter the expression of the two main tight junction proteins- coding genes: occludin and claudin-4. Additionally, TIMP-1 did not affect the expression of associated tight junction protein coding gene, ZO-1. Finally, TIMP-1 did not affect the expression of the adherence junction gene β-catenin.

We then evaluated the effect of TIMP-1 treatment on the protein expression of two important tight junction proteins, i.e., occludin and claudin-4. We demonstrate that occludin degradation was significantly inhibited when Caco-2 cells were exposed to TIMP-1. This finding may have a significant implication on the putative protective effect that TIMP-1 may exert on NEC disease in terms of MMP inhibition. Since

secreted active MMP's were previously demonstrated to play a key destructive role on the intestinal epithelium inducing a higher permeability of the intestinal barrier and the progression of NEC, inhibiting this activity may be of pivotal importance. Interestingly, the expression of the tight junction protein claudin-4 was not affected by TIMP-1 treatment in the range of concentrations tested. This result may signify that MMP-2 plays a differential role in the degradation of different tight junction proteins. Since both occludin and claudin-4 share a structural resemblance; i.e., A tetra-span protein with two extracellular loops similarly located, at the epical and lateral sides, a likely explanation to occludin specificity by MMP-2 would be the differences in the amino acid sequence found between those two proteins. There are some differences on this regard, and in contrast to claudin-4, occludin includes areas rich in tyrosine especially in the first extracellular ring (19). The cleavage activity of MMP-2's is directed toward peptide bonds found in residues within the hydrophobic side chains (16) such as Phe, Leu, Ile, and Tyr. If we take this structural feature into consideration, then occludin provides a much more suitable candidate for MMP-2 degradation. Therefore,

inhibiting MMP-2's activity may specifically inhibit only occludin degradation whereas it will not induce any effect on claudin-4 degradation. Supporting our assumption, a previous study demonstrated that chemical inhibition of MMP's leads to reduced proteolysis of occludin, and reduces the permeability of cellular junctions (27). Supporting this view, it was also recently shown that MMP-2 activity is involved with occludin degradation in a cerebral ischemia model (28). Unlike occludin, claudin-4 which is a member of the claudins that is expressed in the intestine has no unique areas rich in hydrophobic residues and thus it is less expected to be degraded by MMP-2 cleavage. We summarize in the scheme provided in **Figure 6** the potential role MMP-2 and TIMP-1 may play on tight junction integrity during NEC.

We conclude that one of the mechanisms associated with HBM protection against NEC is mediated by TIMP-1, a protein found at high concentrations in preterm HBM, and demonstrated to play a key role in controlling MMP-2 degradation activity, specifically of the tight junction protein occludin, thereby preserving tight junctions integrity and avoiding the destruction of the gut barrier characteristic of NEC disease.

METHODS

This study was approved by The Hebrew University of Jerusalem Review Board.

Cell Culture

The human colon cell line Caco-2 (purchased from the American Type Culture Collection, Manassas, VA), was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 20% (v/v) FBS (SAFC Biosciences Lenexa, KS) and 0.2% (v/v) Penicillin Streptomycin Nystatin (Biolab-chemicals, Jerusalem, Israel). Cells were grown in 37 °C humidified atmosphere 95% air and 5% CO_2.

TIMP-1 Experiments

Confluent Caco-2 cells were trypsinized with a trypsin-ethylenediaminetetraacetic acid solution (Biological Industries, Beit Haemek, Israel), counted and seeded (3×10^5) in 12-well plates (Thermo Fisher Scientific—Nunc A/S, Roskilde, Denmark). After 48 h, the medium was replaced to DMEM supplemented with 0.5% (v/v) FBS and 0.2% (v/v) Penicillin Streptomycin Nystatin. Cells were treated for 12 h with recombinant TIMP-1 (Aviva systems biology, San Diego, CA), that was added to the medium at final concentrations of 10, 50, 100, and 200 ng/ml. Cells not treated with TIMP-1 (exposed only to fresh culture medium), served as control. At the end of the treatment, the CM was collected and kept at −80 °C until used for Zymography analysis. The cells were lysed with RIPA lysis buffer or Tri reagent (Sigma Aldrich) and kept at −80 °C until further analysis.

Zymography

Equal volumes of CM were separated on 10% SDS-PAGE, containing 0.1% (w/v) gelatin (Type A, Sigma Aldrich). The gel was washed twice with washing buffer (Triton X-100 2.5% v/v, Tris pH 7.5), then incubated for 24 h in Tris buffer pH 7.6 containing 10 mmol/l $CaCl_2$, at 37 °C. After incubation, the gel was stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma Aldrich) solution (50% methanol, 7% acetic acid) for 1 h, and then destained in 3% glycerol
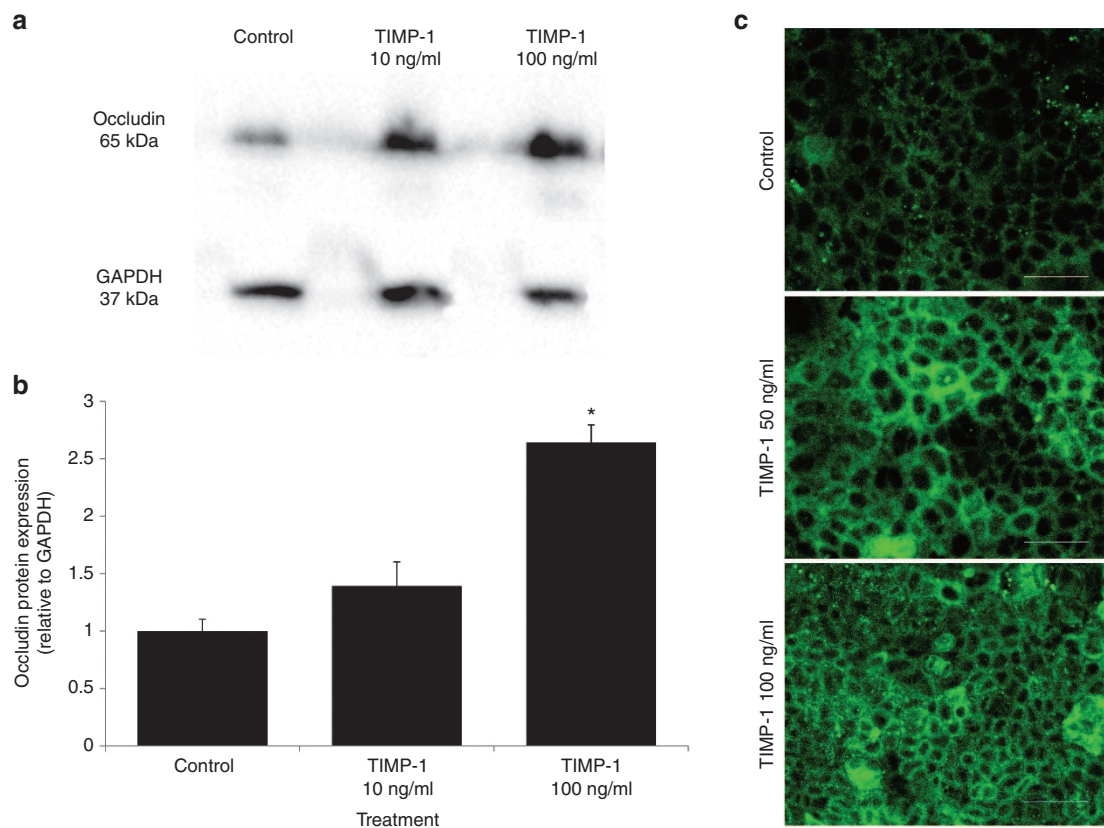

Figure 5. Effect of recombinant TIMP-1 on occludin protein degradation in Caco-2 cells. (**a**) Lysates of control or TIMP-1 (10 and 100 ng/ml) treated cells were used for western blot analysis. (**b**) Densitometry analysis of bands is presented as expression of occludin relative to GAPDH. There was a significant difference between TIMP-1 (100 ng/ml) and control *($P < 0.001$). Data are expressed as mean ± SEM ($n = 12$). (**c**) Immunofluorescence staining was used to verify and visualize changes in occludin expression. Representing photomicrographs of Caco-2 cells stained for occludin (green), scale bar = 50 μm.
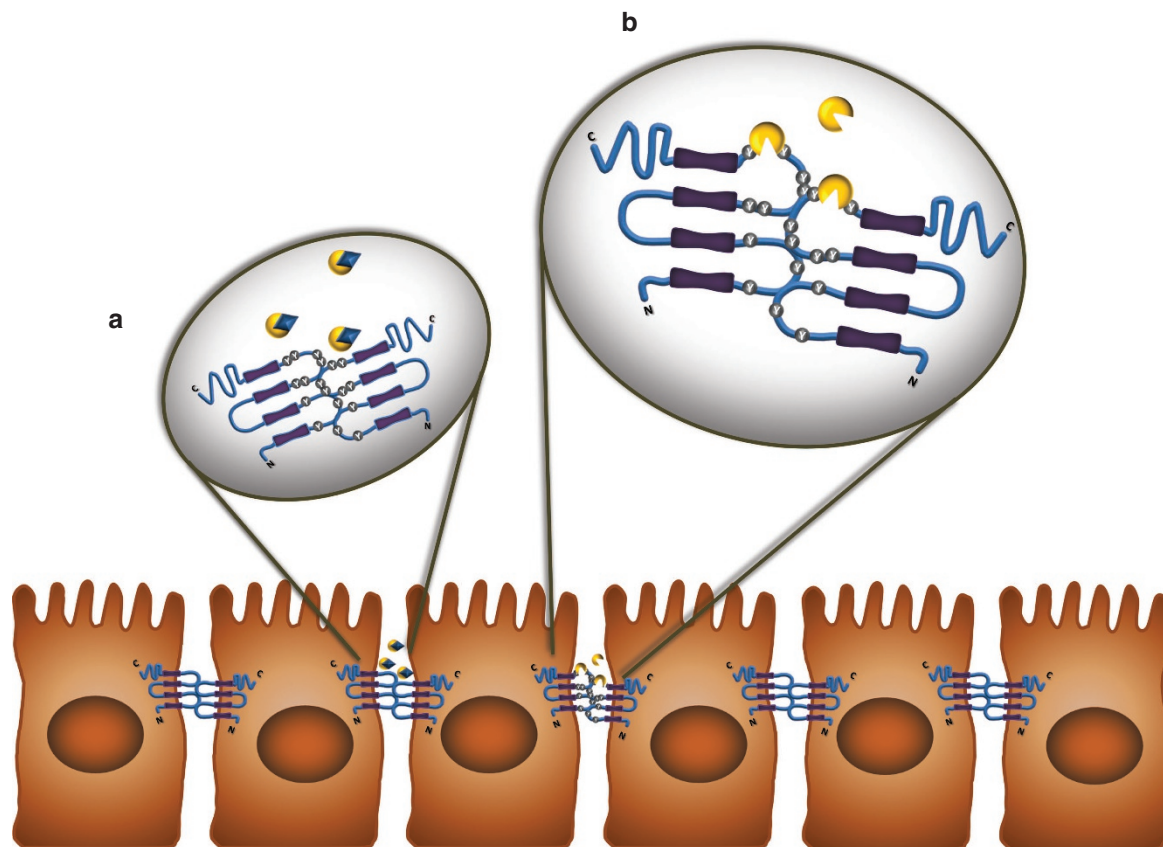
Figure 6. Theoretical model: protective effect of TIMP-1 on epithelial barrier integrity. Our suggested model represents a mechanism by which tight junction protein occludin is protected from degradation by matrix metalloproteinases (MMP-2) through TIMP-1 inhibition of MMP-2's proteolytic activity. This specific inhibition of occludin's degradation exerted by TIMP-1 can promote the maintenance of intact epithelial barrier in NEC, effect possible provided by HBM (from mothers of preterm babies), containing high levels of TIMP-1. Occludin blue-purple twisted shape, TIMP-1 blue diamond, MMP-2 yellow "Pacman", Tyrosine "Y" in solid circle.

solution (containing 20% methanol, 7% acetic acid), and scanned with ChemiDoc MP System (Biorad, Hercules, CA).

Immunofluorescence
Cells were grown on round 18-mm glass cover slips precoated with 0.1% (w/v) gelatin (Type A, Sigma Aldrich), and treated as described above. At the end of the experiment the cells were fixed with 3.7% (w/v) paraformaldehyde solution, washed with phosphate-buffered saline and incubated with anti-Occludin antibody (Life technologies, Carlsbad, CA) over night. After washing (×4) with phosphate-buffered saline, cells were incubated with secondary antibody (Alexa 488, Invitrogen, Life technologies), washed (×4) with phosphate-buffered saline and mounted on glass slides with Flouro-mounting media (Sigma Aldrich). Fluorescent pictures were captured with an Eclipse TE2000-U Confocal microscope (Nikon, Tokyo, Japan).

MMP-2 Inhibition Assay
Inhibition of MMP-2 enzymatic activity by TIMP-1 was assessed by a commercial kit (ENZO Life Sciences, Farmingdale, NY), based on the thiopeptide (Ac-PLG-(2-mercapto-4-methyl-pentanoyl)-LG-OC2H5) as a chromogenic substrate. After preincubation, according to the manufacturer's instructions, the kinetic of MMP-2 inhibition was measured with a spectrophotometer (TriStar LB 941, Berthold Technologies GmbH & Co. KG) at a wavelength of 405 nm taken each at 1 min intervals continuously for 1 h. The effect of different concentrations of recombinant TIMP-1 (ranged from 10 to 200 ng/ml), were tested. The inhibitor NNGH (N-Hydroxy-2-(((4-methoxyphenyl)sulfonyl)(2-methylpropyl)amino)acetamide) provided the activity control. Results (calculated according to the manufacturer's equation and instructions) were obtained from four different measurements that were normalized to blanks and their averages used to calculate the slopes. The values calculated from the slopes were then used to

calculate the percent of the remaining MMP-2 activity relative to control.

Western Blot Analyses
Whole cell lysates (45 μg of protein) were separated in 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated for 1 h in 5% (w/v) skim milk—TBST (Tris-buffered saline supplemented with 0.1% (v/v) Tween 20). Membranes were then incubated over night with primary antibodies against occludin (Life Technologies), Claudin-4 (Santa Cruz Biotechnologies, Dallas, Texas) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies). After washing the membranes with TBST, they were incubated for 1 h at room temperature with corresponding horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). The membranes were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnologies), and photographed with ChemiDoc MP System (Biorad). Quantification of bands was carried out with Image Lab software (Biorad).

Quantitative Real-Time PCR
Total RNA was isolated using TRI Reagent (Sigma Aldrich), according to the manufacturer's protocol. Thousand nanograms of RNA were reverse transcribed to cDNA using the qScript cDNA Synthesis kit (Quanta BioSciences, Gaithersburg, MD). 18.75 ng of cDNA were used for each Sybr green (Quanta BioSciences) Real-Time PCR reaction with the 7300 Real-Time PCR (Applied Biosystems, Life technologies). Primers for these reactions (Hy-Labs, Rehovot, Israel) were designed against known human sequences: Occludin (NM_001205254.1), forward, 5′-GGACTCTACGTGGATCAGTATTTG-3′; reverse, 5′-AAT AATCATGAACCCCAGTACAATG-3′. Claudin-4 (NM_001305.4), forward, 5′-TGGGGCTACAGGTAATGGG-3′; reverse, 5′-GGTCTG

CGAGGTGACAATGTT-3. GAPDH (NM_001256799.1), forward, 5′-CTGGGCTACACTGAGCACC-3′; reverse, 5′-AAGTGGTCGTT GAGGGCAATG-3′. ZO-1 (NM_003257.3), forward, 5′-GGACCA GCTGAAGGACAGCT-3′; reverse, 5′-TCCGTTAACCATTGCAA CTCG-3′. β-Catenin (NM_001098209.1), forward, 5′-CTTGGAC TGAGACTGCTGATCTTG-3′; reverse, 5′-CACCAGAGTGAAAAG AACGATAGCTA-3′. Bax (NM_004324.3), forward, 5′-CAAGAC CAGGGTGGTTGG-3′; reverse, 5′-CACTCCCGCCACAAAGAT-3′. Bcl-2 (NM_000633.2), forward, 5′-TTGACAGAGGATCATGCTG TACTT-3′; reverse, 5′-ATCTTTATTTCATGAGGCACGTT-3′.

Statistical Analysis

Quantitative data were presented as means ± SEM. Statistical significance was evaluated with JMP (SAS Institute, Cary, NC). Results were considered statistically different when $P < 0.05$ by one-way ANOVA followed by Tukey's or Student's *t*-test.

REFERENCES

1. Neu J, Walker WA. Necrotizing enterocolitis. N Engl J Med 2011;364: 255–64.
2. Lin PW, Stoll BJ. Necrotising enterocolitis. Lancet 2006;368:1271–83.
3. Schnabl KL, Van Aerde JE, Thomson AB, Clandinin MT. Necrotizing enterocolitis: a multifactorial disease with no cure. World J Gastroenterol 2008;14:2142–61.
4. Petty JK, Ziegler MM. Operative strategies for necrotizing enterocolitis: The prevention and treatment of short-bowel syndrome. Semin Pediatr Surg 2005;14:191–8.
5. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010;156:562–7.e1.
6. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336:1519–23.
7. Quigley MA, Henderson G, Anthony MY, McGuire W. Formula milk versus donor breast milk for feeding preterm or low birth weight infants. Cochrane Database Syst Rev 2007:CD002971.
8. Meinzen-Derr J, Poindexter B, Wrage L, Morrow AL, Stoll B, Donovan EF. Role of human milk in extremely low birth weight infants' risk of necrotizing enterocolitis or death. J Perinatol 2009;29:57–62.
9. Martin CR, Walker WA. Intestinal immune defences and the inflammatory response in necrotising enterocolitis. Semin Fetal Neonatal Med 2006;11:369–77.
10. Hartsock A, Nelson WJ. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 2008;1778:660–9.
11. Lubetzky R, Mandel D, Mimouni FB, Herman L, Reich R, Reif S. MMP-2 and MMP-9 and their tissue inhibitor in preterm human milk. J Pediatr Gastroenterol Nutr 2010;51:210–2.
12. Sim WH, Wagner J, Cameron DJ, Catto-Smith AG, Bishop RF, Kirkwood CD. Expression profile of genes involved in pathogenesis of pediatric Crohn's disease. J Gastroenterol Hepatol 2012;27:1083–93.
13. Baugh MD, Perry MJ, Hollander AP, et al. Matrix metalloproteinase levels are elevated in inflammatory bowel disease. Gastroenterology 1999;117:814–22.
14. Stallmach A, Chan CC, Ecker KW, et al. Comparable expression of matrix metalloproteinases 1 and 2 in pouchitis and ulcerative colitis. Gut 2000;47:415–22.
15. Pender SL, Braegger C, Gunther U, et al. Matrix metalloproteinases in necrotising enterocolitis. Pediatr Res 2003;54:160–4.
16. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–39.
17. Medina C, Radomski MW. Role of matrix metalloproteinases in intestinal inflammation. J Pharmacol Exp Ther 2006;318:933–8.
18. Bister V, Salmela MT, Heikkilä P, et al. Matrilysins-1 and -2 (MMP-7 and -26) and metalloelastase (MMP-12), unlike MMP-19, are up-regulated in necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 2005;40:60–6.
19. Feldman GJ, Mullin JM, Ryan MP. Occludin: structure, function and regulation. Adv Drug Deliv Rev 2005;57:883–917.
20. Ritter LM, Garfield SH, Thorgeirsson UP. Tissue inhibitor of metalloproteinases-1 (TIMP-1) binds to the cell surface and translocates to the nucleus of human MCF-7 breast carcinoma cells. Biochem Biophys Res Commun 1999;257:494–9.
21. Frost BL, Caplan MS. Necrotizing enterocolitis: pathophysiology, platelet-activating factor, and probiotics. Semin Pediatr Surg 2013;22:88–93.
22. Alfaleh K, Anabrees J, Bassler D, Al-Kharfi T. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev. 2011 16;(3):CD005496.
23. Olson MW, Gervasi DC, Mobashery S, Fridman R. Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. J Biol Chem 1997;272: 29975–83.
24. Verstappen J, Von den Hoff JW. Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease. J Dent Res 2006;85:1074–84.
25. Zhao WQ, Li H, Yamashita K, et al. Cell cycle-associated accumulation of tissue inhibitor of metalloproteinases-1 (TIMP-1) in the nuclei of human gingival fibroblasts. J Cell Sci 1998;111 (Pt 9):1147–53.
26. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609–19.
27. Wachtel M, Frei K, Ehler E, Fontana A, Winterhalter K, Gloor SM. Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. J Cell Sci 1999;112 (Pt 23): 4347–56.
28. Liu J, Jin X, Liu KJ, Liu W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. J Neurosci 2012;32:3044–57.