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Tranexamic acid and the gut barrier: Protection by inhibition of trypsin uptake and activation of downstream intestinal proteases

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ABSTRACT

Objective: Intraluminal pancreatic trypsin and other digestive enzymes injure the gut barrier following trauma-hemorrhagic shock (T/HS). Intestinal proteases (sheddases) exert important effects on normal gut function but may cause barrier disruption due to exaggerated production following T/HS. We hypothesized that the protective mechanism of TXA on the gut barrier following T/HS includes inhibition of these "downstream" proteases. This was studied *in vitro*.

Methods: Trypsin, matrix metalloproteinase (MMP-9) and ADAM-17 activity were measured in intestinal epithelial cells (IEC) exposed to HR + trypsin. TXA was added to IEC subsets. Pulmonary microvascular endothelial cells (HMVEC) were exposed to IEC supernatants and syndecan release and ICAM-1 expression determined.

Results: Trypsin activity and the activity of the "downstream" sheddases ADAM-17, MMP was increased in IEC lysates following exposure to HR + trypsin. Syndecan and ICAM-1 were increased in HMVEC exposed to IEC supernatants. TXA administration 'early' abrogated these effects.

Conclusions: TXA administration early after shock protects the gut barrier by inhibiting trypsin uptake and activity and the subsequent downstream protease cascade. To be effective, TXA should be administered early in all "at risk" patients.

1. Introduction

Trauma/hemorrhagic shock (T/HS) may lead to gut barrier failure and resultant systemic inflammation and remote organ injury. Intraluminal non bacterial intestinal components have an important role in the pathogenesis of T/HS induced gut and lung injury.[1] Studies from the laboratory of Schmid-Schönbein and colleagues[2,3] have demonstrated that during splanchnic ischemia the intestinal mucosal barrier becomes disrupted leading to the entry of pancreatic digestive enzymes into the intestinal wall, initiating "auto-digestion". Subsequently, trypsin and other digestive enzymes may then escape into the systemic circulation activating other proteases which may cause lung and other remote organ dysfunction.[1] These investigators as well as other groups have demonstrated that the intraluminal administration of Tranexamic acid (TXA) and other inhibitors throughout the entire intestine prior to or shortly after splanchnic ischemia insults abrogate subsequent gut and lung injury.[1] This intraluminal therapeutic technique is impractical in the clinical setting.

We therefore studied the intestinal barrier properties of "systemic" TXA administration in an *in vitro* cell culture model. In these studies, we demonstrate preservation of the intestinal mucus and epithelial cell components of the gut barrier subjected to hypoxia-reoxygenation (HR) and trypsin challenge.[4] TXA administration with clinically relevant concentrations was only effective when given within one hour after reoxygenation.[5] The pathogenesis may involve activation of downstream proteases, "sheddases", and lead to lung injury. This was studied in our *in vitro* intestinal epithelial cell model.

2. Materials and methods

2.1. Caco-2 cells

Caco-2 cells were obtained from ATCC (Rockford, MD), No. HTB37 and maintained with DMEM (Gibco, Grand Island, NY). Briefly, confluent monolayers were harvested and a new culture

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flask was started and the remaining cells used to set up co culture in a two-chamber cell culture system (Transwell; Corning Costar Core, Cambridge, MA).

2.2. HT29-MTX cells

The HT29-MTX-E12 cell line was obtained from HPA Cultures (Salisbury, UK) and routinely cultured with DMEM (Gibco, Grand Island, NY). Briefly, confluent monolayers were harvested and a new culture flask was subsequently started and the remaining cells used to set up co culture in a two-chamber cell culture system (Transwell; Corning Costar Core, Cambridge, MA).

2.3. Caco-2/HT29-MTX Co culture

Cultures of 50% Caco-2 and HT29-MTX were seeded into the apical chamber of a two-chamber cell culture system at a final density of 2×10^4 cells/ml and were used for our studies. The formation of a complete cell monolayer was monitored by serial measurement of the transepithelial electrical resistance (TEER) by means of a Millicell electrical resistance system (Millipore, Bedford, MA).

2.4. Pulmonary endothelial cells

Human Pulmonary Microvascular Endothelial Cell's (HMVEC) were obtained from Cambrex Bio Science Walkersville Inc. No. CC-2527 (Walkersville, MD) and routinely cultured with Endothelial Cell Basal Medium-2 (EBM-2; Lonza, Walkersville, MD), supplemented with EGM-2 MV SingleQuots (Lonza, Walkersville, MD). 1×10^5 cells were seeded into the apical chamber of a two-chamber cell culture system. The formation of a complete monolayer was monitored by TEER.

2.5. Experimental design

Intestinal epithelial cell co cultures were exposed to hypoxia followed by reoxygenation. Hypoxic conditions (90 min at 5% O_2; balance N_2, 37 °C) were carried out in a hypoxic glove box purchased from Coy Laboratory Products (Ann Arbor, MI), after which cell culture plates were returned to normal conditions for 90 min (21% O_2, 37 °C). Subgroups were challenged with trypsin (5 μM) added to the apical chamber during the hypoxic event. Tranexamic acid (150 μM) was subsequently added immediately after the hypoxic challenge (T = 0) and 60 and 120 min post hypoxia challenge. Intracellular trypsin activity, downstream "sheddase" proteins and their activity were measured by western blot and ELISA techniques. In separate experiments, HMVEC were exposed to basal chamber co culture supernatants for 2 h at 37 °C and ICAM-1 surface expression and syndecan shedding were measured.

2.6. Measurement of trypsin activity

Trypsin activity in cell lysates was measured with 50 mM substrate specific for trypsin (*N*-Benzoyl-D,L-arginine 4-nitroanilide hydrochloride, BAPNA). Briefly, the initial rates of hydrolysis were measured by the fluorescent intensity of *p*-nitroaniline at 380/460 nm (excitation/emission) which is released upon hydrolysis of the substrate. The initial velocity of the reaction was calculated as the rate of fluorescent units (RFU) per μg of protein per minute. In additional experiments, trypsin was added directly to HMVEC monolayers to assess any effect trypsin may have on HMVEC monolayer integrity directly.

2.7. Matrix metalloproteinase (MMP) analysis using western blot

Proteins in HT29-MTX/Caco-2 monolayer supernatants were separated using polyacrylamide gel by electrophoresis (PAGE). The proteins were subsequently transferred to a nitrocellulose membrane. Membranes were incubated with the primary antibody; rabbit anti-human MMP-9 (Abcam, Cambridge, MA). Membranes were washed and incubated with goat anti-rabbit IgG HRP conjugate (Sigma, St. Louis, MO). After the final wash, the membrane was incubated with Sigma FAST DAB tablets (substrate for HRP) and reaction stopped with TBS. Densitometric measurements of protein bands were detected using Eagle Sight software (STRATAGENE, Inc.).

2.8. MMP activity

The activity of matrix metalloproteinases present in co culture cell lysates was assayed using an MMP Activity Assay Kit (Abcam, Cambridge, MA). Briefly, controls and test samples are mixed with a fluorescence resonance energy transfer (FRET) peptide which serves as a MMP substrate. Upon cleavage of the substrate by MMPs within control and test samples, the fluorescence is recovered and read by a fluorescence microplate reader at excitation = 540 nm and emission = 590 nm. Results are expressed as relative fluorescence units (RFU).

2.9. ADAM-17 ELISA

Co culture basal chamber supernatants were collected and assayed for the presence of the "sheddase" ADAM-17 by ELISA (ThermoScientific, Grand Island, NY). Briefly, ADAM-17 standards and culture supernatants were added to a 96 well plate pre coated with an ADAM-17 specific antibody. 100 μl of a biotinylated antibody is added followed by Streptavidin-HRP. Incubation with TMB substrate follows and development of plate in the dark. Absorbance (450 nm) is measured and ADAM-17 levels calculated.

2.10. ADAM-17 activity

The measurement of ADAM-17 activity was accomplished using the InnoZyme TACE Activity Kit (EMD Millipore, Billerica, MA). Briefly, control and test samples are added to a 96 well microtiter plate pre-coated with a monoclonal antibody specific for human ADAM-17. The activity of captured ADAM-17 enzyme is measured using a fluorescent substrate. The level of fluorescence is measured at an excitation wavelength of 324 nm and emission wavelength of 400 nm. Results are expressed as relative fluorescence units (RFU).

2.11. ICAM-1 molecule quantification

Flow cytometry was used to quantitatively determine the density of ICAM-1 on the surface of HMVEC. Briefly, HMVEC exposed to basal chamber supernatants from HT29-MTX/Caco-2 co cultures were incubated with fluorescein-conjugated anti-CD54 antibody (R&D Systems, Inc., Minneapolis, MN) for 30 min at 4 °C. HMVEC are re-suspended in 400 μl of PBS and cell surface expression of ICAM-1 is determined by flow cytometric analysis using 488 nm wavelength laser excitation. Data is expressed as mean fluorescence intensity (MFI).