Die Fragmentierung der Extrazellulären Matrix als lokaler Trigger für den postoperativen Ileus

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1. Deutsche Zusammenfassung

1.1 Einleitung

Der intestinale postoperative Ileus (POI) ist eine regelmäßig auftretende Komplikation abdominalchirurgischer Eingriffe (Kehlet, 2008). Er ist durch eine, den gesamten Gastrointestinaltrakt (GI-Trakt) betreffende, funktionelle Inhibition des propulsiven Kontraktilitätsmusters gekennzeichnet. Beim Patienten führt diese Darmmotilitätstörung zu einer signifikanten Morbidität mit postoperativer Nausea, Erbrechen, Immobilität, verlängertem Krankenhausaufenthalt und damit erheblichen Hospitalisationskosten (Iyer et al., 2009; Prasad et al., 1999). Eine möglichst schnelle Rehabilitation der physiologischen Darmmotilität stellt daher einen entscheidenden Faktor für den erfolgreichen Verlauf einer chirurgischen Therapie dar.

Vorversuche haben gezeigt, dass der POI durch eine inflammatorische Antwort des Darms ausgelöst wird. Diese besteht aus der Aktivierung eines dichten Netzwerks von Makrophagen in der Darmmuskularis, welche Zytokine (IL-6 = *Interleukin-6*, TNF-α = *tumor necrosis factor alpha*), Chemokine und motilitätshemmende Substanzen (iNOS = *inducible nitric oxide synthase*, COX-2 = *Cyclooxygenase-2*) ausschütten. Dieses lokal-entzündliche Milieu führt zur Rekrutierung von Immunzellen in die zirkuläre Muskelschicht. Diese wiederum halten durch lokale und systemische Sekretion von proinflammatorischen Mediatoren die Entzündung aufrecht und triggern diese. Insgesamt führen diese Mechanismen zu einer herabgesetzten neuromuskulären Kontraktionskraft mit einer gleichzeitigen Aktivierung von neuroinhibitorischen Signalwegen, die postoperativ die Motilität des gesamten GI-Traktes unterdrücken können (Kalff et al., 2000; Kalff et al., 2003; Schwarz et al., 2001; Schwarz et al., 2004; Turler et al., 2002; Turler et al., 2006; Wehner et al., 2005; Wehner et al., 2007).

Es ist von früheren Studien bekannt, dass der POI ein multifaktorielles Ereignis ist, bei dem aber die mechanische intestinale Manipulation (IM) der Darmwand selber eine entscheidende Rolle spielt (Kalff et al., 2003; Schwarz et al., 2004). Die konsekutive Fragmentierung der interstitiellen Extrazellulärmatrix (ECM) führt zur Freisetzung bzw. Aufregulation proinflammatorischer Zellbestandteile und Aktivierung profinflammatorischer Signalkaskaden durch freigesetzte ECM-Fragmente (Buchholz et al., 2009; Heldin et al., 2008; Taylor et al., 2004).

Ziel dieser Studie war es, die pathophysiologische Rolle der Matrix-Metalloproteinasen (MMPs) als Bestandteil der Darmmuskularis in der Signalkaskade des POI zu untersuchen. Zusätzlich sollte die Hyaluronsäure als einer der hauptverantwortlichen Enzyme für den Ab- und Umbau der ECM als signifikante Triggerkomponente einer lokalen und systemischen Entzündungsreaktion, die letztlich im POI nach IM resultiert, identifiziert werden. Diese Erkenntnisse könnten helfen, den deletären Verlauf des POI zu verhindern.

1.2 Material und Methoden

Experimentelle Gruppen und Operationsmodell

Wir verglichen die intestinale Manipulation (IM) mit einer Sham-Operation. Unter Anästhesie mit Isofluran wurden bei Sprague-Dawley-Ratten nach einer Medianlaparotomie Zökum und Dünndarm auf sterile, feuchte Gaze eventriert. Danach erfolgte gemäß Protokoll eine standardisierte IM entlang des gesamten Intestinaltraktes mit sterilen Wattestäbchen (Moore et al., 2005). Nach Rückverlagerung in die Bauchhöhle erfolgte ein zweischichtiger Verschluss der Bauchwand. In der Kontrollgruppe wurde lediglich eine Laparatomie mit Eventration und anschließender Rückverlegung des Darmes ohne IM durchgeführt (Sham-Operation). Die Analyse erfolgte 0, 3, 6, und 24 h nach IM mit der Darmentnahme. Für jede Gruppe und jeden Untersuchungszeitpunkt wurden jeweils 6 Tiere verwendet. Die Tierversuche wurden nach den Richtlinien des Tierschutzgesetzes durchgeführt und der Tierversuchsantrag (Aktenzeichen: 8.87-50.10 / 31.08.076) von der Bezirksregierung Köln genehmigt.

In vivo gastrointestinaler Transit

Der gastrointestinale Transit wurde über die gastrointestinale Verteilung von Flourescein-gebundenem Dextran (90 Minuten nach oraler Bolusgabe) gemessen (Overhaus et al., 2006). Hierzu wurde der Ratte 24 h nach IM das Flourescein-gebundene Dextran über eine Magensonde appliziert. Das geometrische Zentrum (geometric center, GC) diente der Quantifizierung der medianen Verteilung über den GI-Trakt (Overhaus et al., 2006).

ECM-Aktivität in Peritonalflüssigkeit und Serum

0, 3, 6 und 24 h nach IM wurde 8 ml NaCl-Flüssigkeit intraperitoneal injiziert und nach leichter Massage des Abdomens 2 ml für weitere Analysen zurückgewonnen. Zusätzlich wurde zu den gleichen Zeitpunkten venöses Blut aus der *Vena cava inferior* entnommen, zentrifugiert und asserviert. In Peritonealflüssigkeit (PF) und Serum wurden mittels ELISA (*enzyme-linked immunosorbent essay*) und Zymographie die Expressionslevel der ECM-Komponenten MMP-9, TIMP-1 *(tissue inhibitor of metalloproteinases)*, Hyaluronsäure und dem Zytokin IL-6 bestimmt.

mRNA-Messungen in der Muskularis von Dünn- und Dickdarm

Nach der Darmentnahme wurde der gesamte Dünn- und Dickdarm in ca. 5 cm lange Segmente geschnitten. Nach Entfernung des *Mesenteriums* wurde gemäß Protokoll die *Muscularis externa* von der *Mucosa* mittels angefeuchteter Wattestäbchen getrennt, bei -80 °C schockgefroren, asserviert und mittels RT-PCR (*real-time polymerase chain reaction*) auf die Expression von IL-6, CD44 und MMP-9 analysiert (Kalff et al., 1998).

Zymographie

Nach der Aufbereitung der Proben wurde bei 125 Volt auf vorgefertigten Gelatinegelen eine Elektrophorese durchgeführt. Die Färbung erfolgte nach Protokoll der Firma BioRad®. Die Verdauungsgebiete wurden als nicht gefärbte Regionen der Gele visualisiert.

ECM-Aktivierung in der Zellkultur

In einer weiteren Serie von Experimenten wurden Peritonealmakrophagen gemäß Protokoll isoliert. Hierzu wurde den Versuchstieren 8 ml 3 % Thioglycollat intraperitoneal appliziert. Nach 48 h wurde eine Peritoneallavage durchgeführt und daraus die Peritonealmakrophagen isoliert und für 72 h inkubiert. Vor den Versuchen wurde das Zellkultur-Medium mit FCS (*Fetal Calf Serum*)-freiem Medium ersetzt. 12 h später erfolgte die Exposition mit PF, die aus den Tieren 6 h nach IM gewonnen worden war. Dieser Zeitpunkt wurde ausgewählt, da sich dieser in unseren Experimenten als Zeitpunkt der maximalen ECM-Freisetzung gezeigt hatte. Nach 1 h Inkubationszeit wurde der Überstand mit Kulturmedium ersetzt; nach einer weiteren Inkubationszeit von

6 h wurde der Überstand gewonnen und mittels ELISA auf IL-6-Level analysiert. In einer weiteren Reihe von Experimenten wurden die Peritonealmakrophagen mit synthetischer niedermolekularer Hyaluronsäure (<250 kDa) für 6 bzw. 24 h stimuliert. Danach erfolgte die Abschabung der inkubierten Peritonealmakrophagen. Die Analyse erfolgte mittels RT-PCR auf IL-6 und MIP1-α (*macrophage inflammatory protein 1 alpha*).

Statistische Datenanalyse

Die Daten wurden als Mittelwert ± Standard error of the mean (SEM) dargestellt. Zur statistischen Auswertung erfolgte ein ungepaarter *Student's t-Test* oder eine 2-way ANOVA (*analysis of variance*) gefolgt von einem *Bonferroni-Test*. Ein Level von $p \leq 0,05$ wurde als statistisch signifikant erachtet.

1.3 Ergebnisse:

In vivo gastrointestinaler Transit

Während in den naiven Kontrolltieren 24 h nach intragastraler Gabe der Fluoreszenzmarker das terminale Ileum mit einem GC von 9,18 ± 0,67 erreichte, entwickelten die Tiere nach IM eine signifikante, klinisch relevante, postoperative panenterische Dysmotilität. In dieser Gruppe verblieb eine signifikante Menge des Dextrans 24 h nach Manipulation im Magen, der Floureszenzmarker war im Mittel nur bis in den Bereich des mittleren Dünndarms verteilt (GC = 6,92 ± 1,47; $p \leq 0,003$).

ECM-Aktivität in Peritonealflüssigkeit und Serum

Wir nahmen an, dass die IM zu Fragmentierung der ECM führt. Daher wurden Messungen zur MMP-9-mRNA-Expression in der Darmmuskularis und Freisetzung von MMP-9, TIMP-1 und Hyaluronsäure in der PF durchgeführt. Mittels ELISA war ein signifikanter Anstieg der TIMP-1-Freisetzung in die PF und Zirkulation nach IM zu verzeichnen, mit einem Peak in PF und Serum 24 h nach IM (jeweils 12,76-fach und 6,96-fach). Das Hyaluronsäure-Level war in PF und Serum 6 h nach IM mit einer massiven 1139-fachen Freisetzung signifikant aufreguliert; im Serum geringer, aber mit einem 508-fachen Anstieg 6 h nach IM immer noch signifikant erhöht ($p < 0,05$).

mRNA-Messungen in der Muskularis von Dünn- und Dickdarm

Die RT-PCR-Analysen zeigten einen deutlichen Anstieg der MMP-9-mRNA-Spiegel in der Muskularis des Dünndarms (Kontrolle = 1,3 ± 0,4 vs. 3 h nach IM = 8,45 ± 2,29, vs. 6 h nach IM = 30,0 ± 10,7 und vs. 24 h nach IM = 37,4 ± 6,5, x-facher Anstieg vs. Kontrolle; $p < 0,05$) und Dickdarms (Kontrolle = 1,1 ± 0,1 vs. 3 h nach IM = 23,0 ± 10,1, vs. 6 h nach IM = 30,1 ± 9,5 und 24 h nach IM = 91,4 ± 5,4, x-facher Anstieg vs. Kontrolle; $p < 0,05$). Es zeigte sich auch eine schnelle und anhaltende Aufregulation der CD44-mRNA-Expression, mit maximaler Induktion 6 h nach IM im Dünndarm (11,3-fach; $p < 0,05$) und nach 3 h im Dickdarm (3,4-fach; $p < 0,05$). In der Muskularis des manipulierten Dünn- und Dickdarms war der Entzündungsmarker IL-6 zu allen Zeitpunkten aufreguliert, die maximale Induktion der mRNA lag bei 6 h nach IM (jeweils 220,4- und 125,8- fach; $p < 0,05$).

Zymographie

Analog zu der erhöhten Transkription konnte auch eine erhöhte Aktivität von MMP-9 mittels Zymographie in der PF nach IM im Vergleich zur Kontrolle gezeigt werden (2,96-, 3,33-, und 1,37- facher Anstieg der Enzymaktivität 3, 6, und 24 h nach IM).

ECM-Aktivierung in der Zellkultur

In dieser Versuchsreihe untersuchten wir das inflammatorische Potential von hyaluronsäurereicher PF auf kultivierte Makrophagen. Während in den Zellkultur-experimenten die Inkubation von frisch isolierten Peritonealmakrophagen mit PF von Tieren nach Sham-Prozedur zu einer messbaren basalen Sekretion von IL-6 ins Zellmedium führte (27,4 ± 1,52 pg/ml), war bei Exposition mit PF, die aus Tieren 6 h nach IM gewonnen worden war, eine signifikant höhere Freisetzung von IL-6 ins Zellmedium nachzuweisen (201,7 ± 0,73 pg/ml; $p < 0,05$). 6 h und 24 h nach Exposition mit synthetischer Hyaluronsäure zeigten die RT-PCR-Messungen der Peritonealmakro-phagen einen jeweils 25,6- und 24,4-fachen Anstieg in der IL-6 ($p < 0,05$), und einen 2,7- und 2,24-fachen Anstieg der MIP1-α-mRNA-Expression ($p < 0,05$).

1.4 Diskussion

Die Familie der Matrix-Metalloproteinasen (MMPs) spielt nicht nur eine große Rolle im Matrix-Turnover, sondern auch in der Aktivierung und beim Abbau von Zyto- und Chemokinen (Medina et al., 2006; Nagase et al., 2006). Der Einfluss von proinflammatorischen Zytokinen auf die postoperative Darmmotilität wurde schon in vielen Studien demonstriert, der Zusammenhang zur Extrazellulären Matrix bisher aber noch nicht dargestellt.

Unsere Daten zeigen jetzt erstmalig, dass es zeitabhängig nach intestinaler Manipulation zu einer signifikanten Induktion von MMP-9-mRNA in der Dünndarm- und Dickdarmmuskularis kommt. Gleichzeitig zeigt sich auch ein Anstieg der aktiven MMP-9-Form in der PF der manipulierten Tiere. Diese Aufregulation der MMP-9 sowohl in der Darmmuskularis, als auch in der PF führt in Folge zu einem verstärkten ECM-Abbau in der Darmwand und über eine Freisetzung von proinflammatorisch wirkenden ECM-Fragmenten und Zellbestandteilen aus der manipulierten Darmwand zu einer lokalisierten und systemischen konsekutiven Aktivierung proinflammatorischer Signalkaskaden. Für den Prozess der MMP-9-Aufregulation könnte teilweise die Freisetzung von Hyaluronsäure während der Darmmanipulation verantwortlich sein, da Hyaluronsäure bekannterweise an der Konvertierung von MMP-2 und MMP-9 in ihre aktive Form beteiligt ist (Isnard et al., 2001). Es kann daher diskutiert werden, dass die Hochregulation von MMP-9 ein Resultat der ECM-Fragmentierung nach IM ist.

Parallel zur MMP-9-Hochregulation wurde auf Proteinebene in der PF und im Serum ebensfalls eine Hochregulation des endogenen Inhibitors der Metalloproteinasen TIMP-1 beobachtet. Dieser spielt auch in anderen Krankheitsbildern eine wichtige, gegenregulatorische Rolle (Medina et al., 2006; Nagase et al., 2006). Wir vermuten, dass diese Aufregulation als antiinflammatorische Antwort zu erwarten ist, um die Schwere des POI zu begrenzen, so wie es auch bei Patienten mit schwerer Sepsis vermutet wird (Lorente et al., 2009).

Hyaluronsäure ist ein ubiquitär vorkommendes Molekül und ein wesentlicher Bestandteil der ECM, das als Polymer synthetisiert wird. Es ist bekannt, das während Gewebeschäden, Entzündungsreaktionen und Beseitigung von Zellmaterial, fragmentierte

Hyaluronsäure durch erhöhte MMP-Aktivität in die Zirkulation freigesetzt wird (Wang et al., 1996). Es ist zudem gezeigt worden, dass fragmentierte Hyaluronsäure in Zellkulturbedingungen direkt proinflammatorisch auf Peritonealmakrophagen via Aktivierung von nf-ϰB (*nuclear factor ϰB*) und auf Endothelzellen via den TLR4 (*toll-like receptor*) Signalweg wirken kann (Noble et al., 1996). Interessanterweise induziert Hyaluronsäure in seiner niedermolekularen Form die Proliferation und Migration von inflammatorischen Zellen, während die hochmolekulare Form > 250 kDa eine inhibitorische Wirkung hat (Bot et al., 1998). Zudem ist CD44 ein ubiquitär vorkommendes Glykoprotein und gleichzeitig ein Rezeptor für Hyaluronsäure. Es ist an Zelladhäsion und Leukozytenrekrutierung an Entzündungsorte beteiligt (Buchholz et al., 2009). Bei der Entwicklung einer Entzündung kann Hyaluronsäure Zellmotilität und -proliferation regulieren, indem es an den CD44-Rezeptor bindet und intrazelluläre Signalkaskaden auslöst (McKee et al., 1997; Rockey et al., 1998).

Die massive Hochregulation von Hyaluronsäure in unserem Versuchsaufbau nach IM in PF und Serum mit gleichzeitiger Hochregulation seines Rezeptors CD44 bestärkt uns in der Annahme, dass niedermolekulare Hyaluronsäure als ECM-Zerfallsprodukt eine wichtige Rolle in der synergistischen Triggerung inflammatorischer Signalwege in Makrophagen der Darmmuskularis nach IM spielt (Buchholz et al., 2009; Taylor et al., 2004). Diese resultieren über die Ausschüttung einer großen Menge an Entzündungsmediatoren (IL-6, IL-1β, TNF-α, MCP-1, iNOS, COX-2, MMPs) in einer Entzündungsreaktion im Darm und folglich in einer Darmmotilitätseinschränkung (Bauer et al., 2008; Kalff et al., 1998; Schwarz et al., 2004; Turler et al., 2002; Turler et al., 2006).

Diese Hypothese konnte in dieser Studie auch dadurch untermauert werden, dass in kultivierten Peritonealmakrophagen, die einerseits mit synthetisch hergestellter niedermolekularer Hyaluronsäure oder andererseits mit PF, die aus Tieren nach IM gewonnen wurde, stimuliert worden waren, signifikant erhöhte Proteinlevel für das proinflammatorische IL-6 und und eine signifikante Hochregulation von IL-6 und MIP1-α (Chemokin mit proinflammatorischen Eigenschaften, das von Makrophagen produziert wird) mRNA nachzuweisen waren. Auch in der Darmmuskularis zeigte sich eine Hochregulation von IL-6. Diese Ergebnisse geben uns wichtige Informationen bezüglich der profinflamma-

torischen Rolle der fragmentierten intestinalen ECM als Trigger für Makrophagen-aktivierung in der Darmwand und der konsekutiven Entwicklung eines POI.

Es sind sicherlich noch weitere wichtige Zusammenhänge zu erheben, insbesondere welche genauen Mechanismen der ECM-verstärkten postoperativen Entzündungs-antwort zugrunde liegen. Ein weiterer Ansatz zum Anschluss an diese Studie wäre z.B. die Durchführung o.g. Versuche an MMP-9-Knockout-Tieren. Die MMP-9-Inhibition kann einen neuen Therapieansatz darstellen, um die Morbidität des POI zu begrenzen.

Zusammenfassend zeigt diese Studie, dass es durch IM postoperativ zu einer erhöhten Transkription und Aktvitität von MMP-9 mit konsekutiver Freisetzung von niedermolekularen proinflammatorisch wirkenden Hyaluronsäurefragmenten und gleich-zeitiger Induktion des Hyaluronsäurerezeptors, CD44, in der Darmmuskularis kommt. Diese Ereignisse nehmen an der Triggerung einer intestinalen proinflammatorischen Entzündungsantwort teil, die als Antwort auf die Manipulation des Darms in einem POI resultieren.

1.5 Literaturverzeichnis der deutschen Zusammenfassung

Bauer AJ. Mentation on the immunological modulation of gastrointestinal motility. Neurogastroenterology and motility: the official journal of the European Gastrointestinal Motility Society 2008; 20: 81-90

Bot PT, Hoefer IE, Piek JJ, Pasterkamp G. Hyaluronic acid: targeting immune modulatory components of the extracellular matrix in atherosclerosis. Current medicinal chemistry 2008; 15: 786-791

Buchholz BM, Chanthaphavong RS, Bauer AJ. Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. Journal of immunology 2009; 183: 6744-6753

Heldin P, Karousou E, Bernert B, Porsch H, Nishitsuka K, Skandalis SS. Importance of hyaluronan-CD44 interactions in inflammation and tumorigenesis. Connective tissue research 2008; 49: 215-218

Isnard N, Legeais JM, Renard G, Robert L. Effect of hyaluronan on MMP expression and activation. Cell biology international 2001; 25: 735-739

Iyer S, Saunders WB, Stemkowski S. Economic burden of postoperative ileus associated with colectomy in the United States. Journal of managed care pharmacy 2009; 15: 485-494

Kalff JC, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. Gastroenterology 2000; 118: 316-327

Kalff JC, Schraut WH, Simmons RL, Bauer AJ. Surgical manipulation of the gut elicits an intestinal muscularis inflammatory response resulting in postsurgical ileus. Annals of surgery 1998; 228: 652-663

Kalff JC, Schwarz NT, Walgenbach KJ, Schraut WH, Bauer AJ. Leukocytes of the intestinal muscularis: their phenotype and isolation. Journal of leukocyte biology 1998; 63: 683-691

Kalff JC, Turler A, Schwarz NT, Schraut WH, Lee KK, Tweardy DJ, Billiar TR, Simmons RL, Bauer AJ. Intra-abdominal activation of a local inflammatory response within the human muscularis externa during laparotomy. Annals of surgery 2003; 237: 301-315

Kehlet H. Postoperative ileus - an update on preventive techniques. Nature clinical practice Gastroenterology & Hepatology 2008; 5: 552-558

Lorente L, Martin MM, Labarta L, Diaz C, Sole-Violan J, Blanquer J, Orbe J, Rodríguez JA, Jiménez A, Borreguero-León JM, Belmonte F, Medina JC, Llimiñana MC, Ferrer-Agüero JM, Ferreres J, Mora ML, Lubillo S, Sánchez M, Barrios Y, Sierra A, Páramo JA. Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. Critical care (London, England) 2009; 13: 158

McKee CM, Lowenstein CJ, Horton MR, Wu J, Bao C, Chin BY, Choi AM, Noble PW. Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. The Journal of biological chemistry 1997; 272: 8013-8018

Medina C, Radomski MW. Role of matrix metalloproteinases in intestinal inflammation. The Journal of pharmacology and experimental therapeutics 2006; 318: 933-938

Moore BA, Overhaus M, Whitcomb J, Ifedigbo E, Choi AM, Otterbein LE, Bauer AJ. Brief inhalation of low-dose carbon monoxide protects rodents and swine from postoperative ileus. Critical care medicine 2005; 33: 1317-1326

Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovascular research 2006; 69: 562-573

Noble PW, McKee CM, Cowman M, Shin HS. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. The Journal of experimental medicine 1996; 183: 2373-2378

Overhaus M, Moore BA, Barbato JE, Behrendt FF, Doering JG, Bauer AJ. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. American journal of physiology Gastrointestinal and liver physiology 2006; 290: 695-703

Prasad M, Matthews JB. Deflating postoperative ileus. Gastroenterology 1999; 117: 489-492

Rockey DC, Chung JJ, McKee CM, Noble PW. Stimulation of inducible nitric oxide synthase in rat liver by hyaluronan fragments. Hepatology 1998; 27: 86-92

Schwarz NT, Kalff JC, Turler A, Engel BM, Watkins SC, Billiar TR, et al. Prostanoid production via COX-2 as a causative mechanism of rodent postoperative ileus. Gastroenterology 2001; 121: 1354-1371

Schwarz NT, Kalff JC, Turler A, Speidel N, Grandis JR, Billiar TR, et al. Selective jejunal manipulation causes postoperative pan-enteric inflammation and dysmotility. Gastroenterology 2004; 126:159-169

Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. The Journal of biological chemistry 2004; 279: 17079-17084

Turler A, Kalff JC, Moore BA, Hoffman RA, Billiar TR, Simmons RL, Bauer AJ. Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. Annals of surgery 2006; 244: 220-229

Turler A, Moore BA, Pezzone MA, Overhaus M, Kalff JC, Bauer AJ. Colonic postoperative inflammatory ileus in the rat. Annals of surgery 2002; 236: 56-66

Turler A, Schwarz NT, Turler E, Kalff JC, Bauer AJ. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. American journal of physiology Gastrointestinal and liver physiology 2002; 282: 145-155

Wang P, Ba ZF, Biondo A, Chaudry I. Liver endothelial cell dysfunction occurs early following hemorrhagic shock and persists despite crystalloid resuscitation. The Journal of surgical research 1996; 63: 241-247

Wehner S, Behrendt FF, Lyutenski BN, Lysson M, Bauer AJ, Hirner A, Kalff JC. Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. Gut 2007; 56: 176-185

Wehner S, Schwarz NT, Hundsdoerfer R, Hierholzer C, Tweardy DJ, Billiar TR, Bauer AJ, Kalff JC. Induction of IL-6 within the rodent intestinal muscularis after intestinal surgical stress. Surgery 2005; 137: 436-446

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Iatrogenic extracellular matrix disruption as a local trigger for postoperative ileus

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ABSTRACT

Background: Active matrix metallopeptidase 9 (MMP-9) disruption of the extracellular matrix (ECM) plays an important role in inflammatory disorders. In this study, we investigated the inflammatory role of MMP-9 and the ECM breakdown product hyaluronan as a trigger for the postoperative intestinal inflammatory response of postoperative ileus.

Methods: We performed a standardized intestinal surgical manipulation on rats to produce ileus assessed by the oral non-digestible fluorescein isothiocyanate–dextran transit assay. We studied isolated intestinal muscularis extracts for mRNA expressions of interleukin 6 (IL-6), MMP-9 and CD44. We quantified peritoneal MMP-9 activity using zymography, and quantified peritoneal fluid and serum for hyaluronan and tissue inhibitor of metalloproteinase 1 levels by enzyme-linked immunosorbent assay (ELISA). We cultured peritoneal macrophages and exposed them to peritoneal fluid or synthetic hyaluronan for ELISA analysis of IL-6 and macrophage inflammatory protein-1α.

Results: Transit was significantly delayed after surgical manipulation, and extracts of the isolated jejunal and colonic muscularis demonstrated a significant induction of IL-6, MMP-9, and CD44 mRNAs compared with controls. Zymography confirmed significant MMP-9 activity in peritoneal fluid compared with controls. Enzyme-linked immunosorbent assay measurements showed a significant up-regulation in hyaluronan and tissue inhibitor of metalloproteinase 1 in the peritoneal fluid and serum. In addition, ELISA and reverse transcriptase–polymerase chain reaction measurements of peritoneal macrophages stimulated with postsurgical peritoneal fluid and synthetic hyaluronan resulted in higher expressions of IL-6 and macrophage inflammatory protein-1α in the macrophage supernatant.

Conclusions: Our results confirm that MMP-9 disruption in the ECM with hyaluronan release and muscularis CD44 receptor induction has the potential to trigger muscularis proinflammatory cascades that cause postoperative ileus. Matrix metallopeptidase 9 inhibition may be a novel therapeutic approach to limit postoperative ileus.

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1. Introduction

The morbidity of postoperative intestinal atony and ileus is widely acknowledged, and its economic burden has been estimated in the United States to be $1 billion annually [1]. Recent studies have shown that prolonged postoperative intestinal ileus is incited by the generation of an enteric molecular inflammatory response that consists of the activation of the dense network of resident muscularis macrophages and their secretion of cytokines (interleukin 6 [IL-6] and tumor necrosis factor α), chemokines, and smooth muscle inhibitory substances via inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). This local inflammatory milieu then causes the increased expression of vascular adhesion molecules and a subsequent recruitment and extravasation of leukocytes into the circular muscle layer, with a further release of potent leukocytic products that perpetuate intestinal atony. Together, these events succeed in delaying gastrointestinal transit, decrease local neuromuscular function, and activate neurogenic inhibitory pathways that suppress motility along the entire gastrointestinal tract for sustained postoperative periods [2–9].

We know from earlier studies that anesthesia, laparotomy, or bowel eventration do not produce a prolonged ileus, but that the physical manipulation of the gut wall itself is necessary to elicit this detrimental response [3,4]. This suggests that physical manipulation of the gastrointestinal tract leads to a disruption in the interstitial extracellular matrix (ECM), which maintains the cohesiveness of the numerous cellular constituents of the muscularis externa. The aims of this study were to analyze intestinal ECM breakdown via matrix metallopeptidase 9 (MMP-9) activity, and to explore the potential of its breakdown product hyaluronan as an inflammatory trigger that participates in the postoperative inflammatory response generated by the dense network of normally quiescent muscularis macrophages, which leads to the deleterious state of postoperative ileus.

The key role of MMP-9 and tissue inhibitor of metalloproteinase 1 (TIMP-1) in the progression, prognosis, and treatment of gastrointestinal cancers is being intensively investigated [10,11]. A significant body of evidence has also developed in the inflammatory bowel disease literature over the past decade, indicating that increased mucosal MMP-9 activity during episodes of inflammation participates in the destruction of the epithelial barrier, which subsequently exposes the immunologically active lamina propria to bacterial antigens, resulting in the production of proinflammatory mediators that further aggravate the pathology [12,13]. Interestingly, ECM fragments of hyaluronan have been shown to be directly proinflammatory in cell culture conditions on peritoneal macrophages via nuclear factor-κB activation [14] through CD44 receptor binding and on endothelial cells through *TLR4* ligand activity [15].

To our knowledge, however, no previous study has focused on the pathophysiological role of MMP-9 activity on the ECM within the intestinal muscularis in any disease. In this study, we show that manipulation-induced postoperative ileus is associated with a rapid and sustained induction of MMP-9 activity within the postsurgical muscularis externa, which results in a dramatic increase in peritoneal hyaluronan levels bathing the gut wall. Furthermore, the receptor for hyaluronan (CD44) is postoperatively induced and postoperative peritoneal fluid and exogenous hyaluronan possess significant proinflammatory potential on cultured peritoneal macrophages. These data indicate that limiting the proinflammatory activity of MMP-9–generated hyaluronan may be a novel approach to limiting the morbidity of postoperative ileus.

2. Methods

2.1. *Animals*

We purchased Sprague-Dawley male rats (280–320 g) from Charles River Laboratories (Sulzfeld, Germany) and maintained them in a pathogen-free animal facility at the University of Bonn with standard rat chow and tap water supplied ad libitum. They were allowed to acclimatize at least 5 d before experimental manipulation. The District Government of Köln, Germany, approved the animal protocol.

2.2. *Intestinal manipulation*

We subjected the entire intestine of the animals to a standardized, moderate surgical manipulation (SM) as described previously [16]. In brief, we anesthetized animals with continuous isoflurane inhalation (DeltaSelect, Pfullingen, Germany) and performed a midline abdominal incision. We eventrated the cecum and small bowel, placed them onto moist gauze outside the abdominal cavity, and kept them moist with saline. Next, we manipulated the entire small bowel and colon using moist sterile cotton applicators in a standardized fashion. After manipulation, we replaced the intestine and closed the laparotomy with two layers of continuous sutures. Age-matched, non-manipulated, naive animals without surgery served as controls ($n = 6$/group).

2.3. In vivo *gastrointestinal transit*

We measured gastrointestinal transit in controls and manipulated animals 24 h postoperatively by evaluating the gastrointestinal distribution of fluorescein-labeled dextran (molecular weight = 70,000; Sigma-Aldrich, Munich, Germany) as previously described [17]. For statistical analysis, we calculated a geometric center (GC) for the median distribution of fluorescein-labeled dextran along the gastrointestinal tract as previously described [17].

2.4. *Peritoneal fluid and serum*

Postoperatively, after 0, 3, 6, and 24 h, we obtained peritoneal fluid (2 mL/animal) and preserved it–at −20°C ($n = 6$). In brief, we injected sterile saline intraperitoneally at the defined time points after abdominal surgery in manipulated or control animals and recollected and analyzed peritoneal fluid for ECM components and cytokines. We withdrew venous blood samples (8 mL/animal) from the different animal groups at the defined time points from the inferior vena cava, centrifuged

them (3000 rpm for 5 min), and preserved 2 mL of serum for further measurements.

2.5. Enzyme-linked immunosorbent assay (ELISA) measurement

We performed ELISA (R&D Systems, Wiesbaden, Germany) for hyaluronan and tissue inhibitor of metalloproteinases (TIMP-1) expression levels in the peritoneal fluid and in the serum according to the manufacturer's instruction and analyzed them in a Tecan Saphire microplate reader (Tecan Germany GmbH, Crailsheim, Germany). We measured hyaluronan using an ELISA kit, which allows analysis and measurement of hyaluronan in humans and animals, and is a sandwich protein binding assay in a microplate format. The assay uses microwells coated with a highly specific hyaluronic acid binding protein from bovine cartilage to capture hyaluronan, and an enzyme-conjugated version of hyaluronic acid binding protein to detect and measure hyaluronan.

2.6. Muscularis preparation for mRNA measurements

After harvesting the rat's intestine, we cut the entire small intestine and colon into approximately 5-cm segments. We pinned each segment in a Sylgard coated dish (Dow Corning, Wiesbaden, Germany) to remove the attached mesentery. Next, we stripped off the muscularis externa and separated it from the mucosa using moist cotton applicators. We snap-froze the muscularis tissue samples and stored them at −80°C. We performed mRNA real-time reverse transcriptase–polymerase chain reaction for IL-6, CD-44, and MMP-9 expression levels in the intestinal muscularis (small bowel and colon) at 3, 6, and 24 h after SM ($n = 6$/group).

2.7. mRNA expression in intestinal muscularis

We quantified mRNA expressions using the RNA II Extraction kit (Macherey-Nagel, Düren, Germany) followed with rDNAse treatment for DNA digest (Macherey-Nagel). We performed cDNA synthesis with the high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany) in the UNO thermal block (ThermoScientific, Karlsruhe, Germany). We performed polymerase chain reaction using a Universal PCR Master Mix, No AmpErase (Applied Biosystems), and measured and analyzed it with a TaqMan assay for 40 cycles (Applied Biosystems). Table 1 lists the sequences of the TaqMan assays (IL-6, CD44, and MMP-9).

Table 1 – Taqman assays of nucleotide sequences of the rat.

Gene symbol	Assay identification	Reference sequence	Amplicon length
18s-rRNA	HS99999901_s1		187
IL-6	Rn00561420_m1	NM_012589.1	128
CD44	Rn00681157_m1	NM_012924.2	71
MMP-9	Rn00579162_m1	NM_031055.1	72
MIP-1α	Rn00564660_m1	NM_013025.2	76

2.8. Zymography

We analyzed peritoneal fluid and serum samples for MMP-2 and MMP-9 activity. We prepared each sample by dilution into zymogram sample buffer (Bio-Rad Laboratories GmbH, Munich, Germany) and loaded it into the wells of a precast gel containing 0.1% gelatin (Invitrogen, Karlsruhe, Germany). We carried out electrophoresis at 125 V constant current for 1.5–2 h, until the bromphenol blue dye of the sample buffer reached the bottom of the gel. We removed and incubated the gel for 1 h at room temperature in development buffer (Bio-Rad Laboratories GmbH) in a rotary shaker. Next, after decantation, we replaced the development buffer with enzyme buffer (Bio-Rad Laboratories GmbH) and incubated it at 37°C for 18 h. We carried out staining with Coomassie Blue-250 (Bio-Rad Laboratories GmbH) and destaining out at room temperature on a rotary shaker. We visualized areas of digestion as non-staining regions of the gel. Values are expressed in pixels per background minus the digested area.

2.9. Cell culture

We intraperitoneally injected rats with 8 mL 3% thioglycollate medium (Brewer thioglycollate Medium; Sigma-Aldrich, Munich, Germany). After 48 h, we performed peritoneal lavage and harvested macrophages in the peritoneal fluid. In brief, we injected rats with 50 mL harvest medium and lightly massaged them. After 3 min, we extracted about 30–40 mL of medium from the intraperitoneal cavity and put it in two Falcon tubes (Labomedic, Bonn, Germany). We performed this procedure twice. We centrifuged the Falcon tubes and isolated peritoneal macrophages. After isolating, we counted cells using a Neubauer hemocytomer (Assistant, Sondheim, Germany) plated in culture flasks with Dulbecco's modified Eagle's medium buffer (Lonza, Cologne, Germany) and fetal calf serum (FCS) (Lonza), and placed them in a CO_2 incubator (3 million cells/well/six-well plate) for 72 h. Before experiments, we exchanged cell culture medium with FCS free medium, and after 12 h we exposed it to peritoneal fluid. After 1 h of incubation with peritoneal fluid, we removed the supernatant fluid and substituted it with fresh culture medium. After an additional 6 h of incubation, we took the new supernatant fluid, stored it, and analyzed it by ELISA for IL-6 (R&D Systems, Wiesbaden, Germany). We also exposed FCS free macrophages to synthetic low-molecular weight hyaluronan (<250 kDa) (50 μg/mL) dissolved in Dulbecco's modified Eagle's medium buffer for 6 and 24 h. We measured IL-6 and macrophages inflammatory protein (MIP)-1α mRNAs in extracts of the incubated peritoneal macrophages that we collected from the wells, centrifuged, stored, and extracted for PCR Taqman assays (Applied Biosystems) (Table 1).

2.10. Statistical analysis

Results are expressed as means ± standard error of the mean. We performed statistical analysis using unpaired Student's t-test for single comparisons or analysis of variance (ANOVA) for multiple comparisons using the Bonferroni post hoc test. We considered a probability level of $P \leq 0.05$ to be statistically significant.

3. Results

3.1. In vivo *gastrointestinal transit*

We established that our animals developed a clinically relevant postoperative pan-enteric dysmotility by measuring *in vivo* 90-min gastrointestinal transit after bolus oral fluorescein isothiocyanate–dextran administration in naive, non-manipulated controls and manipulating animals 24 h after surgery. At 24 h after surgical manipulation, the transit distribution histograms demonstrated a significant delay in propulsive gastrointestinal motility relative to controls. In naive control animals, the fluorescent marker reached the terminal ileum reflected by a calculated GC of 9.18 ± 0.67, whereas after SM, a significant amount of dextran remained in the stomach, with the marker being distributed mainly throughout the mid-small bowel (GC = 6.92 ± 1.47; $P \leq 0.003$) (Fig. 1).

3.2. *ECM activity in peritoneal fluid and serum*

We hypothesized that SM of the gut wall and the ensuing events results in a disruption in the ECM. Therefore, we measured the expression of MMP-9 mRNA and the release of MMP-9, TIMP-1, and hyaluronan into the peritoneal fluid and serum at 3, 6, and 24 h after SM of the rat gastrointestinal tract. The PCR analysis detected a brisk rise in the expression of MMP-9 mRNA within muscularis extracts of the manipulated small and large intestines at all times investigated (small intestine: control = 1.3 ± 0.4 versus SM-3 h = 8.45 ± 2.29, SM-6 h = 30.0 ± 10.7, and SM-24 h = 37.4 ± 6.5 relative fold increase over control) (large intestine: control = 1.1 ± 0.1 *versus* SM-3 h = 23.0 ± 10.1, SM-6 h = 30.1 ± 9.5, and SM-24 h = 91.4 ± 5.4 relative fold increase over control) (Fig. 2A).

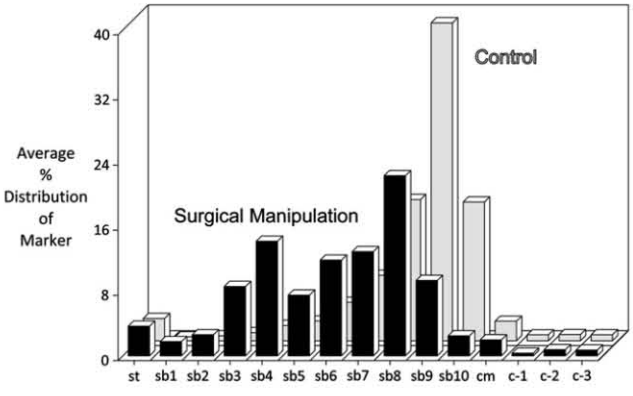

Fig. 1 – Surgical manipulation of the rat gastrointestine produced a postoperative delay in gastrointestinal transit as measured by the luminal distribution of an orally fed bolus of a non-digestible, nonabsorbable fluorescein isothiocyanate-dextran (70 kD) after 90 min. The calculated GC from the individual distribution histograms averaged 9.18 ± 0.67 for control, whereas after SM, the fluorescent marker was mainly distributed within the mid-jejunum and had an average GC of 6.92 ± 1.47 ($n = 6$; $P \leq 0.003$, one-way ANOVA with post hoc Bonferroni analysis).

Correlating with the increase in MMP-9 mRNA, electrophoretic zymography demonstrated a rapid and sustained increase in peritoneal fluid MMP-9 activity after SM. Densitometry quantification of the digested gelatin showed a 2.96-, 3.33-, and 1.37-fold increase in enzymatic activity at 3, 6, and 24 h after SM, respectively. Fig. 2B shows a representative zymography gel. A parallel progressive increase in the release of TIMP-1 into both the peritoneum and the circulation was associated with a persistent rise in MMP-9 at each of the three times assessed (Fig. 3). Although it was already significant at 3 h, we observed the largest fold increase in TIMP-1 24 h after manipulation compared with control peritoneal fluid and serum levels (12.76-fold and 6.96-fold, respectively).

As a consequence of the increase in enzymatic activity on the ECM, further experiments revealed the rapid and significant release of hyaluronan into the peritoneal cavity and circulation after SM (Fig. 4). In the peritoneal fluid, the level of hyaluronan increased 3.01-fold 3 h after SM, but at 6 h after SM, we detected a massive 1139-fold release with a sustained 3.77-fold increase at 24 h. Serum hyaluronan levels were significantly lower compared with the local peritoneal fluid, but as in the peritoneal fluid, we measured a significant increased release of hyaluronan in serum, with a peak 508-fold increase after 6 h.

We next sought to determine the expression level of CD44 mRNA within the muscularis, because CD44 is the membrane receptor for hyaluronan. The PCR analysis of intestinal muscularis extracts demonstrated that the receptor mRNA was promptly and persistently up-regulated over the 24 h of assessment, with the maximal induction occurring at the 6-h point (11.3-fold) for the small intestine and at 3 h for the colon (3.4-fold) (Fig. 5).

3.3. *Inflammatory potential of hyaluronan*

We sought to explore the inflammatory potential of the hyaluronan-rich peritoneal fluid. Interleukin-6 is a prototypical proinflammatory mediator that was significantly induced in this study within muscularis extracts of the surgically manipulated small intestine and colon at all three times investigated. A maximal induction of IL-6 mRNA occurred after 6 h (220.4- and 125.8-fold, respectively). In a series of cell culture experiments, we pre-incubated isolated peritoneal macrophages in culture for 1 h with peritoneal fluid collected from controls or animals 6 h after SM. We chose the 6-h harvest point of the peritoneal fluid after surgery because the above experiments demonstrated it to be the point of maximum ECM fragment release. Incubation of freshly isolated peritoneal macrophages for 6 h exposed to a sham procedure of media exchange led to a measurable basal secretion of IL-6 protein (27.4 ± 1.52 pg/mL) into the media. On the other hand, 6-h harvested SM peritoneal fluid caused a significantly higher release of IL-6 protein (201.7 ± 0.73 pg/mL) into the media compared with control peritoneal fluid pre-incubation macrophages. These data indicate that the hyaluronan-rich postoperative peritoneal fluid possesses inflammatory properties.

In a further series of experiments, we stimulated cultured peritoneal macrophages directly with synthetic low-molecular-weight hyaluronan to determine the sole inflammatory potential of hyaluronan on macrophages. Reverse transcriptase–polymerase chain reaction demonstrated a prolonged

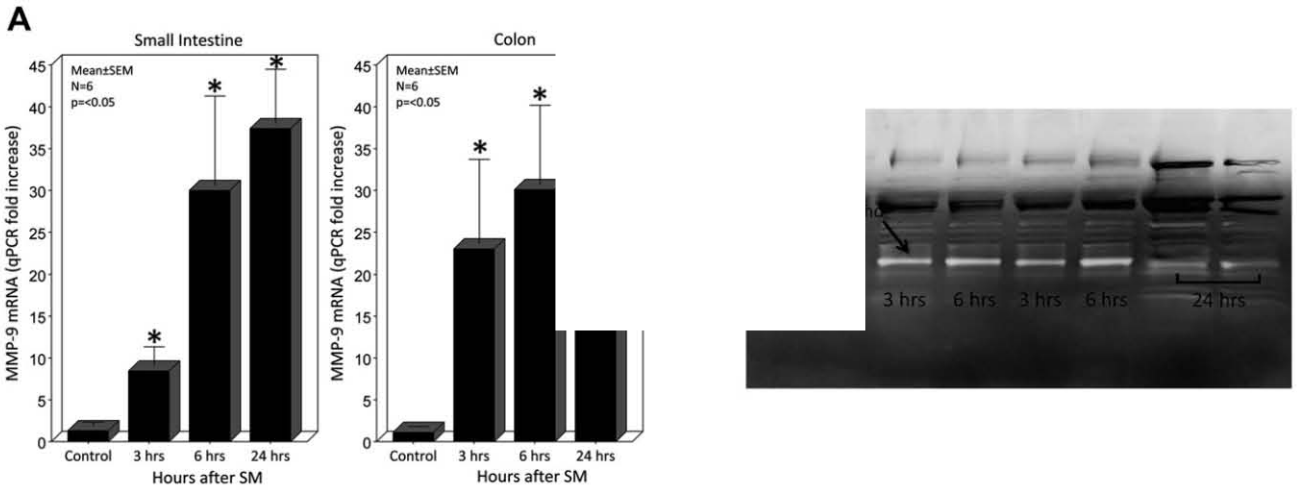

Fig. 2 – (A) Surgical manipulation of the rat gastrointestinal tract initiated a temporal progressive increase in the qPCR expression of MMP-9 mRNA within the small intestinal (left) and colonic (right) isolated muscularis externa. The MMP-9 mRNA was significantly increased at all times measured between 3 and 24 h after SM compared with control (N = 6; *= P < 0.05, one-way ANOVA with post hoc Bonferroni analysis). (B) Representative electrophoretic zymography gel experiment in which a significant increase in MMP-9 enzymatic activity could be demonstrated in the peritoneal fluid harvested 3, 6, and 24 h after SM.

25.6- and 24.4-fold increase in IL-6 mRNA after exposure of the cultured macrophages to hyaluronan for 6 and 24 h, respectively. In addition, hyaluronan caused a 2.7- and 2.24-fold increase in MIP-1α, a factor produced by macrophages that causes local inflammatory responses and induces superoxide production by neutrophils at 6 and 24 h, respectively (Fig. 6). Together, these data indicate that ECM fragments of hyaluronan released from the SM intestine participate in generating the complex inflammatory milieu that exists within the immunologically active postoperative muscularis externa.

4. Discussion

Postoperative intestinal ileus is a common and almost obligatory feature of general visceral surgery [18]. Unfortunately, postoperative ileus results in significant patient morbidity, increased duration of hospital stays, and substantial hospitalization costs [19]. It has been established that an intestinal inflammatory response to SM of the gastrointestinal tract activates proinflammatory pathways, which initiate a cascade

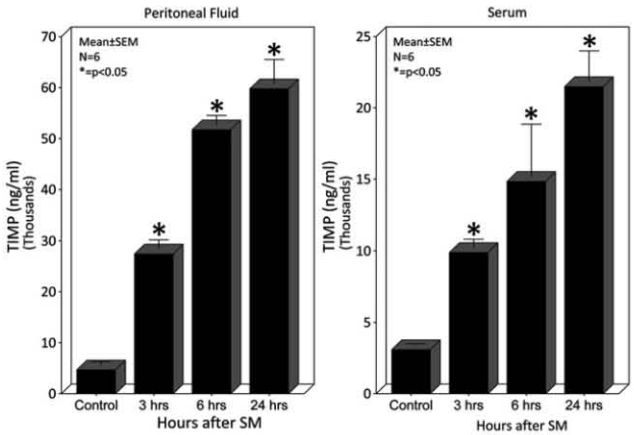

Fig. 3 – Microplate ELISA measurement of TIMP-1 protein levels was performed on peritoneal fluid and serum harvested from controls and SM rats 3, 6, and 24 h after resuscitation. The TIMP-1 protein levels significantly increased rapidly within 3 h and progressively increased through 24 h in both the peritoneal fluid and serum harvested postoperatively (N = 6; *= P < 0.05, one-way ANOVA with post hoc Bonferroni analysis).

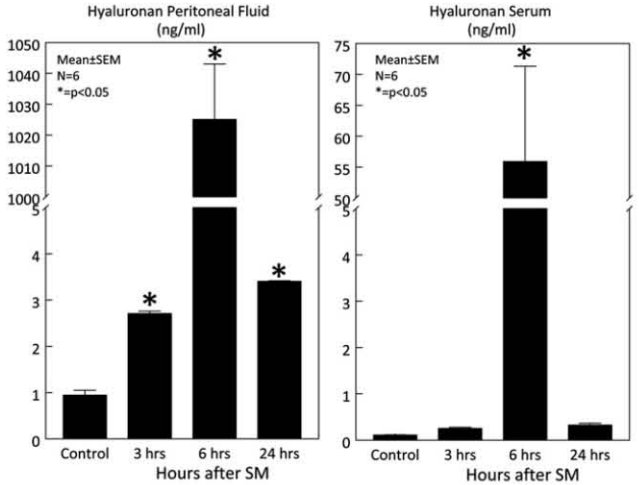

Fig. 4 – Microplate ELISA measurement of hyaluronan levels performed on peritoneal fluid and serum harvested from controls and SM rats 3, 6, and 24 h after resuscitation. Hyaluronan levels significantly increased rapidly within 3 h, peaked at 6 h, and waned but remained elevated through 24 h in the peritoneal fluid. Serum hyaluronan levels significantly increased 6 h postoperatively (N = 6; *= P < 0.05, one-way ANOVA with post hoc Bonferroni analysis).

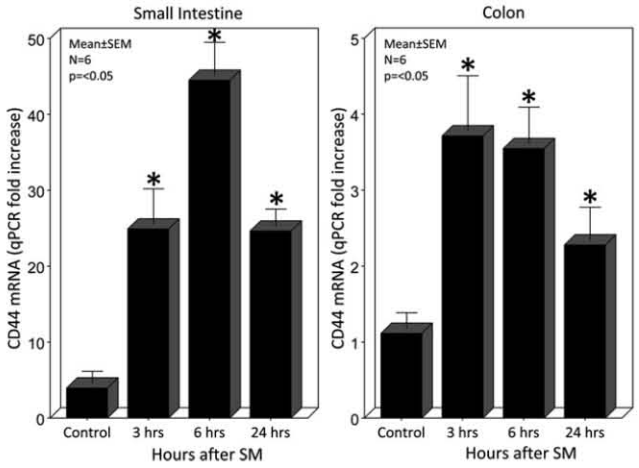

Fig. 5 – The classical hyaluronan receptor, CD44, was transcriptionally induced within the muscularis of the small and large intestines by SM of the gastrointestinal tract. The CD44 mRNA levels significantly increased rapidly within 3 h, peaked at 6 h, and waned but remained elevated through 24 h in the small intestinal muscularis externa. Colonic muscularis CD44 mRNA was also postoperatively rapidly induced at 3 h and gradually declined, but remained elevated through 24 h postoperatively (N = 6; * = P < 0.05, one-way ANOVA with post hoc Bonferroni analysis).

of events characterized by impaired gastrointestinal transit, decreased muscular and neuromuscular function, and increased neurogenic inhibitory activity along the entire gastrointestinal tract [5,6,20,21]. In this study, we investigated the role of MMPs and the muscularis extracellular matrix as a significant triggering component that could initiate and maintain postoperative local molecular and cellular inflammatory responses after intestinal manipulation that produce postoperative ileus.

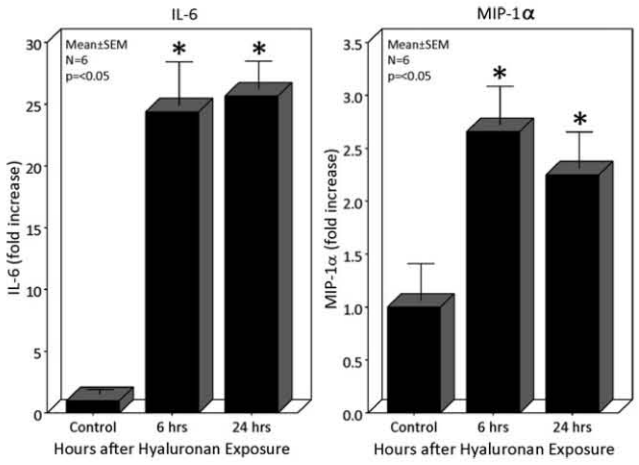

Fig. 6 – Real-time PCR analysis of IL-6 and MIP-1α mRNAs from peritoneal macrophage extracts after exposure to hyaluronan (50 μg/mL) for 6 or 24 h. Hyaluronan caused a significant sustained induction of both IL-6 and MIP-1α in cultured peritoneal macrophages compared with control (N = 6; * = P < 0.05, one-way ANOVA with post hoc Bonferroni analysis).

Matrix metalloproteinases constitute a family of enzymes capable of degrading various ECM and basement membrane components with a role in matrix turnover. They also activate and degrade signaling molecules such as cytokines and chemokines. Matrix metalloproteinases are involved in inflammation and have been implicated in tissue degradation [22,23]. As in other organs, MMPs appear to have an important role in intestinal proinflammatory signaling cascades, as has been previously demonstrated for Crohn's disease [24]. In addition, CD44 and active proteolytic MMP-9 are associated with migrating cells and can mediate collagen IV degradation and promote cell invasion.

In support of the hypothesized important role of the ECM in postoperative ileus, our data demonstrate that MMP-9 mRNA is significantly induced within the muscularis of the small bowel and colon in a time-dependent manner after intestinal manipulation. In addition, we measured a rapid and sustained dramatic increase of the active form of MMP-9 protein in peritoneal fluid after SM of the bowel wall. Part of the rapid increase in activated MMP-9 protein could be due to the release of hyaluronan during gut manipulation itself, because hyaluronan is known to convert the inactive forms of MMP-2 and MMP-9 to their active forms [25]. Hence, MMP activation could occur through mechanical disruption of the matrix, increased activation, and up-regulated gene transcription.

Parallel to the postsurgical increased induction and activity of MMP-9, we investigated the endogenous tissue inhibitor of MMPs, TIMP-1, which has an important counter-regulatory role during various disease states [22,23]. In an effort to understand the inhibitory role of TIMP-1 within the post-surgical degradation of the intestinal ECM, we measured the significant increased expression of TIMP-1 in serum and peritoneal fluid. An augmentation in TIMP-1 activity would be expected to function as an anti-inflammatory response limiting the severity of postoperative ileus, as has been hypothesized in patients with severe sepsis [26].

Hyaluronan is a ubiquitous molecule and a major component of the pericellular matrix and ECM that is synthesized into an extensive polymer. However, its molecular weight is variable. It is known that during tissue injury, inflammation, and clearance of cellular debris, fragmented hyaluronan is released into the circulation by increased MMP activity [27]. Throughout the development of injury, hyaluronan regulates cell motility, invasion, and proliferation by binding to CD44 receptors and activating intracellular signaling pathways. Serum elevations in hyaluronan have been measured in many inflammatory disorders, and the fragmented ECM components (hyaluronan and fibronectin) themselves have been shown to function as proinflammatory stimuli to macrophages [28,29]. Interestingly, the inflammatory properties depend on the size of the polymer. Low-molecular-weight hyaluronan has been shown to be pr-inflammatory and stimulatory for cell proliferation and migration, whereas high-molecular-weight hyaluronan is inhibitory [30].

With this understanding, our results demonstrate a 1000-fold up-regulation in released hyaluronan 6 h after SM in the serum and peritoneal fluid compared with controls. This observation strengthens our premise that low-molecular-

weight hyaluronan as an ECM breakdown product has an important role in triggering the postoperative inflammatory signal cascade via CD44 or *TLR4* on resident intestinal muscularis macrophages and invading leukocytes that subsequently cause the functional impairment of the gastrointestinal tract [15,31]. In support of this hypothesis, we were able to provoke a significant proinflammatory response in cultured peritoneal macrophages stimulated with synthetic low-molecular-weight hyaluronan compared with controls. In addition, we observed a significant increase in IL-6 transcription along with IL-6 and MIP-1α protein secretion in the cultured macrophages and their supernatant after exposure to peritoneal fluid harvested from manipulated animals, compared with fluid harvested from controls. The fact that incubated peritoneal macrophages can be stimulated and activated with synthetic low-molecular-weight hyaluronan and also with peritoneal fluid of manipulated animals provides crucial information regarding the proinflammatory role of the disrupted intestinal ECM, especially as a trigger for macrophage activation and the development of postoperative ileus.

The hyaluronan receptor CD44 is a multifunctional, ubiquitously expressed glycoprotein that participates in cell adhesion and leukocyte recruitment to sites of inflammation by its ligands glycosaminoglycan and hyaluronan [32]. During the development of injury, hyaluronan regulates cell motility, invasion, and proliferation by binding to CD44 receptors and activating intracellular signaling pathways [33]. In our study, the enhanced expression of CD44 mRNA in the intestinal muscularis and the parallel local and systemic release of its ligands (fragmented hyaluronan) from the intestinal ECM of the SM intestine strongly suggest their potential role in synergistically triggering known proinflammatory cascades in intestinal macrophages, which participate in causing the generation of copious amounts of gut-derived inflammatory mediators (IL-6, IL-1β, tumor necrosis factor α, MCP-1, iNOS, COX-2, and MMPs), mucosal barrier function breakdown (iNOS), and ileus (iNOS and COX-2) with the leakage of luminal toxic products [5,7,20,21,34]. Clearly, important mechanistic links remain to be discovered explaining how the ECM-augmented postoperative inflammatory response actually causes ileus. Nevertheless, as we have previously shown, iNOS and COX-2 significantly contribute to postoperative bowel dysfunction.

This study demonstrates that intestinal manipulation results in the increased transcription and activity of MMP-9, and an increase in the release of hyaluronan along with the transcriptional induction of its receptor CD44, and that hyaluronan has the potential to function as a trigger for macrophage activation. Together, these molecular events are hypothesized to participate in the synergistic triggering of the intestinal inflammatory response that is a key element in the intestinal response to the surgeon's hand, resulting in postoperative ileus.

Acknowledgments

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REFERENCES

[1] Prasad M, Matthews JB. Deflating postoperative ileus. Gastroenterology 1999;117:489.
[2] Kalff JC, Schraut WH, Billiar TR, et al. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. Gastroenterology 2000; 118:316.
[3] Kalff JC, Turler A, Schwarz NT, et al. Intra-abdominal activation of a local inflammatory response within the human muscularis externa during laparotomy. Ann Surg 2003;237:301.
[4] Schwarz NT, Kalff JC, Turler A, et al. Prostanoid production via COX-2 as a causative mechanism of rodent postoperative ileus. Gastroenterology 2001;121:1354.
[5] Schwarz NT, Kalff JC, Turler A, et al. Selective jejunal manipulation causes postoperative pan-enteric inflammation and dysmotility. Gastroenterology 2004; 126:159.
[6] Turler A, Moore BA, Pezzone MA, et al. Colonic postoperative inflammatory ileus in the rat. Ann Surg 2002;236:56.
[7] Turler A, Kalff JC, Moore BA, et al. Leukocyte-derived inducible nitric oxide synthase mediates murine postoperative ileus. Ann Surg 2006;244:220.
[8] Wehner S, Schwarz NT, Hundsdoerfer R, et al. Induction of IL-6 within the rodent intestinal muscularis after intestinal surgical stress. Surgery 2005;137:436.
[9] Wehner S, Behrendt FF, Lyutenski BN, et al. Inhibition of macrophage function prevents intestinal inflammation and postoperative ileus in rodents. Gut 2007;56:176.
[10] Mook OR, Frederiks WM, Van Noorden CJ. The role of gelatinases in colorectal cancer progression and metastasis. Biochim Biophys Acta 2004;1705:69.
[11] Yasui W, Oue N, Aung PP, et al. Molecular-pathological prognostic factors of gastric cancer: A review. Gastric Cancer 2005;8:86.
[12] Bailey CJ, Hembry RM, Alexander A, et al. Distribution of the matrix metalloproteinases stromelysin, gelatinases A and B, and collagenase in Crohn's disease and normal intestine. J Clin Pathol 1994;47:113.
[13] Garg P, Vijay-Kumar M, Wang L, et al. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. Am J Physiol Gastrointest Liver Physiol 2009;296:G175.
[14] Noble PW, McKee CM, Cowman M, et al. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. J Exp Med 1996; 183:2373.
[15] Taylor KR, Trowbridge JM, Rudisill JA, et al. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. J Biol Chem 2004;279:17079.
[16] Moore BA, Overhaus M, Whitcomb J, et al. Brief inhalation of low-dose carbon monoxide protects rodents and swine from postoperative ileus. Crit Care Med 2005;33:1317.
[17] Overhaus M, Moore BA, Barbato JE, et al. Biliverdin protects against polymicrobial sepsis by modulating inflammatory mediators. Am J Physiol Gastrointest Liver Physiol 2006; 290:G695.
[18] Kehlet H. Postoperative ileus—an update on preventive techniques. Nat Clin Pract Gastroenterol Hepatol 2008;5:552.
[19] Iyer S, Saunders WB, Stemkowski S. Economic burden of postoperative ileus associated with colectomy in the United States. J Manag Care Pharm 2009;15:485.

[20] Bauer AJ. Mentation on the immunological modulation of gastrointestinal motility. Neurogastroenterol Motil 2008; 20(Suppl 1):81.
[21] Kalff JC, Schraut WH, Simmons RL, et al. Surgical manipulation of the gut elicits an intestinal muscularis inflammatory response resulting in postsurgical ileus. Ann Surg 1998;228:652.
[22] Medina C, Radomski MW. Role of matrix metalloproteinases in intestinal inflammation. J Pharmacol Exp Ther 2006;318:933.
[23] Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 2006; 69:562.
[24] Makitalo L, Sipponen T, Karkkainen P, et al. Changes in matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) expression profile in Crohn's disease after immunosuppressive treatment correlate with histological score and calprotectin values. Int J Colorect Dis 2009;24:1157.
[25] Isnard N, Legeais JM, Renard G, et al. Effect of hyaluronan on MMP expression and activation. Cell Biol Int 2001;25:735.
[26] Lorente L, Martin MM, Labarta L, et al. Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. Crit Care 2009;13:R158.
[27] Wang P, Ba ZF, Biondo A, et al. Liver endothelial cell dysfunction occurs early following hemorrhagic shock and persists despite crystalloid resuscitation. J Surg Res 1996; 63:241.
[28] McKee CM, Lowenstein CJ, Horton MR, et al. Hyaluronan fragments induce nitric-oxide synthase in murine macrophages through a nuclear factor kappaB-dependent mechanism. J Biol Chem 1997;272:8013.
[29] Rockey DC, Chung JJ, McKee CM, et al. Stimulation of inducible nitric oxide synthase in rat liver by hyaluronan fragments. Hepatology 1998;27:86.
[30] Bot PT, Hoefer IE, Piek JJ, et al. Hyaluronic acid: targeting immune modulatory components of the extracellular matrix in atherosclerosis. Curr Med Chem 2008;15:786.
[31] Buchholz BM, Chanthaphavong RS, Bauer AJ. Nonhemopoietic cell TLR4 signaling is critical in causing early lipopolysaccharide-induced ileus. J Immunol 2009;183: 6744.
[32] Johnson P, Ruffell B. CD44 and its role in inflammation and inflammatory diseases. Inflamm Allergy Drug Targets 2009; 8:208.
[33] Heldin P, Karousou E, Bernert B, et al. Importance of hyaluronan-CD44 interactions in inflammation and tumorigenesis. Connect Tissue Res 2008;49:215.
[34] Turler A, Schwarz NT, Turler E, et al. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. Am J Physiol Gastrointest Liver Physiol 2002; 282:G145.

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