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Intestinal mast cells mediate gut injury and systemic inflammation in a rat model of deep hypothermic circulatory arrest

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Abstract

Background—Cardiac surgery, especially when employing cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA), is associated with systemic inflammatory responses that significantly affect morbidity and mortality. Intestinal perfusion abnormalities have been implicated in such responses, but the mechanisms linking local injury and systemic inflammation remain unclear. Intestinal mast cells (MC) are specialized immune cells that secrete various preformed effectors in response to cellular stress. We hypothesized that MCs are activated in a microenvironment shaped by intestinal ischemia/reperfusion (I/R), and investigated local and systemic consequences in a rat model of DHCA.

Methods and Results—Adult rats were cooled to 16–18°C on CPB before instituting DHCA for 45 minutes. Specimens were harvested following rewarming and 2 hours of recovery. Significant intestinal barrier disruption was found, together with macro- and microscopic evidence of I/R injury in ileum and colon but not in lungs or kidneys. Immunofluorescence and toluidine blue staining revealed MC hyperplasia and activation in the gut. Pretreatment with the MC stabilizer cromolyn sodium efficiently blocked MC degranulation and resulted in preserved intestinal morphology and barrier function following DHCA. Furthermore, cromolyn sodium treatment dramatically reduced intestinal neutrophil influx and systemic release of various proinflammatory cytokines.

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Conclusions—Our data provides primary evidence that intestinal I/R is a leading pathophysiologic process in a rat model of DHCA and that intestinal injury and local and systemic inflammatory responses are critically dependent on MC activation. This identifies intestinal MCs as central players in DHCA-associated responses, and opens novel therapeutic possibilities for patients undergoing this procedure.

Introduction

Cardiovascular surgery requiring deep hypothermic circulatory arrest (DHCA) is associated with significant hospital morbidity and mortality.^{1, 2} In this setting, exposure to the cardiopulmonary bypass (CPB) circuit, abrupt alterations in body temperature and whole-body ischemia/reperfusion (I/R) lead to profound perturbations in inflammatory, hemostatic, and oxidative stress pathways, all implicated in the pathogenesis of an often pronounced systemic inflammatory response syndrome (SIRS). This perioperative SIRS has been associated with hemodynamic instability, bleeding disorders, and myocardial, respiratory, renal, and cognitive dysfunction.³

As many aspects of the pathophysiology of SIRS remain unclear, our efforts to prevent or treat SIRS or its progression to multi-organ failure have remained inadequate. In this context, growing interest has focused on the possibility that the gut is the insidious source of systemic inflammation. In the gut, a single-layer epithelial barrier controls compartmentalization of gut-associated inflammatory cells, which collectively form the body's largest immune organ from enormous numbers of luminal microbiota. The anoxic lumen of the gut and the complexity of the underlying vascular bed provide an anatomical setting that makes the intestinal epithelium highly susceptible to perfusion abnormalities. In a number of disease models, barrier dysfunction and epithelial injury develop in a microenvironment of closely interrelated hypoxic and inflammatory pathways.⁴⁻⁶ Ensuing translocation of bacteria and their products has widely been regarded as the most important consequence of intestinal barrier breakdown (reviewed in⁷). However, the immense population of immune competent cells within the gut wall suggests that inflammation is triggered in a more complex manner.

Mast cells (MCs) constitute only 2%-3% of lamina propria⁸ cells, but are perfectly equipped and positioned at the host/environment interface to serve as first responders to invading pathogens and cellular stress signals. Instant release of MC-specific proteases and proinflammatory mediators, such as tumor necrosis factor alpha (TNF- α) and interleukin 6 (IL-6), from intracellular stores are not only critical in local tissue injury but can also rapidly elicit systemic effects, due to the close proximity of MCs to blood vessels.^{8, 9}

It is now well recognized that MC activation is a prominent feature of intestinal I/R, where MC products mediate intestinal injury^{10, 11} as well as distal organ damage.¹² Consequently, we hypothesize that, in a setting of DHCA-associated splanchnic malperfusion, intestinal MC activation contributes to the breakdown of the epithelial barrier and to stimulation of systemic inflammation. In a rat model of DHCA, we therefore examined the role of MCs as a crucial and largely overlooked link between intestinal and systemic inflammatory responses and, as such, a potential target for novel therapeutic intervention.

Methods

Rat DHCA model

All procedures performed for this study were approved by the Duke University Animal Care and Use Committee, and conformed to NIH guidelines for animal care.

Fasting adult male Sprague-Dawley rats (436.5 ± 34 g, 10-12 weeks old) underwent deep hypothermic arrest in association with CPB (referred to in this paper as DHCA for simplicity) as previously described.¹³ Briefly, animals were anesthetized with isoflurane (2-2.5 Vol%), intubated, and mechanically ventilated (45% O₂/balance N₂, PaCO₂ 35-45 mmHg). The tail artery and right external jugular vein were then cannulated, and 150 IU heparin and 5 µg fentanyl were administered. To allow for later immunolocalization of hypoxic tissues, rats received 60 mg/kg pimonidazole hydrochloride (HPI, Inc., Burlington, MA) through the venous access line. Baseline physiologic measurements, including mean arterial pressure, pericranial and rectal temperature, and blood gases, were recorded 10 minutes before CPB was instituted. Animals were then cooled for 30 minutes. Arterial blood gases were maintained using the pH strategy (PaCO₂ 31-40 mmHg).

At a pericranial temperature of 16-18°C, the bypass machine was stopped. Circulatory arrest was maintained for 45 minutes. For rewarming, CPB was reinitiated and at a pericranial temperature 35.5°C, animals were separated from CPB. Animals remained ventilated under anesthesia ("recovery") for 2 hours until euthanasia.

Two animal groups served as experimental controls: sham-treated animals were anesthetized, cannulated, and heparinized but did not undergo CPB; another cohort underwent CPB and hypothermia but not circulatory arrest (deep hypothermic cardiopulmonary bypass [DHCPB]). Experimental times were the same for all experimental groups. A subset of animals in each experimental group received intraperitoneal injections of 50 mg/kg cromolyn sodium (diluted in physiological saline) at 16 hours before the experiment, after induction of anesthesia, and immediately before reperfusion.

Histology and immunohistochemistry

Frozen sections of tissues fixed with Carnoy's fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) were stained with toluidine blue. Formalin-fixed and paraffin-embedded tissue samples were used for histopathological examinations and TUNEL staining. Hematoxylin and eosin-stained sections were assessed by independent observers blinded to treatment modalities.

Renal and pulmonary injury were graded on scales outlined by Jablonski et al¹⁴ and Egan et al,¹⁵ respectively. For mesenteric windows, the supporting mesentery of an ileal loop was mounted onto a slide and immediately immersed in Carnoy's fixative overnight. To determine the percentage of degranulated MCs from the total numbers of identified MCs, 6 independent microscopic fields were analyzed by an examiner blinded to treatment modalities.

For immunohistochemistry, tissue frozen in OCT was cut into 5- μ m-thick sections and fixed in cold acetone. After blocking with normal donkey serum, sections were incubated overnight with one of the following antibodies: rabbit polyclonal carboxypeptidase A3 antibody (1:100; Abbiotec, San Diego, CA), rabbit polyclonal tryptase antibody (1:100; Santa Cruz Biotechnology, CA), or mouse monoclonal anti-Hypoxypore (1:50; HPI). TRITC-labeled avidin (1:1000; Sigma Chemical Company, St. Louis, MO) was also used for MC staining. Nuclear counterstaining (DAPI containing mounting medium; Vector Laboratories, Burlingame, CA) and epithelial immunostaining with a mouse monoclonal E-cadherin antibody (1:200; BD Biosciences, San Jose, CA) provided further anatomic orientation. FITC and CY3-coupled species-specific donkey secondary antibodies (1:1000; Jackson Laboratories, West Grove, PA) were applied for visualization.

Tissue collection and assays

Ussing chamber measurements were performed as previously described.¹⁶ In summary, segments of ileum, caecum, and proximal colon were harvested, and the seromuscular layer was mechanically removed. Tissues were mounted in Ussing chambers with 0.3 cm² apertures and immersed in oxygenated Ringer solution (95% O₂, 5% CO₂) containing 10 mM glucose on the serosal side and 10 mM mannitol on the mucosal side. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp. After stabilizing the preparation, FITC-dextran (2.2 mg/mL, 4.4 kDa; Sigma) was added to the mucosal reservoir. Samples were harvested from the serosal reservoir at 15-minute intervals for 180 minutes, and the concentration of fluorescein was determined by spectrophotofluorometry (CytoFluo 2300, Millipore, Bedford, MA). Results were recorded as the movement of FITC-dextran over time (mg FITC-dextran/min).

For epithelial-enriched fractions, 3-4-cm segments of colon were opened and washed in phosphate-buffered saline (PBS). The mucosal side was then mechanically scraped, and the material obtained was snap frozen. Myeloperoxidase (MPO) activity was measured in snap frozen tissue according to the method described by Weiss et al.¹⁷ In brief, tissue was weighted and homogenized in potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma). Myeloperoxidase activity in the supernatant was determined by following the oxidation of o-dianisidine in presence of hydrogen peroxide (Sigma).

To assess lipid peroxidation, tissues were homogenized in an ice-cold solution of 5 mM butylated hydroxytoluene in PBS. Malondialdehyde (MDA) and 4-hydroxyalkenals were measured together according to the manufacturer's instructions (Oxis International Inc., Foster City, CA).

RNA was isolated from epithelial-enriched fractions using a commercially available kit (Macherey-Nagel, Bethlehem, PA), which includes a DNase digestion step and RNA was reverse transcribed (BioRad, Hercules, CA). Quantitative PCR for the following mRNAs was performed on a CFX96 Real-Time PCR Detection System (BioRad): MC protease-1 (MCP-1), -5, -6, -7, carboxypeptidase A3 (CPA3), glucose transporter-3 (Glut3), CD73,

multiple drug transporter 1 (MDR1), and TNF- α . β -Actin served as internal standard. Primer and PCR conditions are specified in the supplemental data section.

Serum collection and assays

Blood samples were collected after induction of anesthesia, 5 minutes after completion of DHCA, and immediately prior to euthanasia. Following centrifugation at 3000 rpm for 10 minutes at 4°C, serum was stored at -80°C for future analysis. Rat cytokines were quantified using a multiplex ELISA kit (Millipore, Billerica, MA), which allowed simultaneous detection of GRO-KC, IFN- γ , IL-1 β , MCP-1, MIP-1 α , RANTES, TNF- α , IL-10, IL-12 (p70), IL-4, and IL-6. Cytokine binding to the fluorescent-coded beads was detected using the Bio-Plex system (Bio-Rad, Hercules, CA). Plasma histamine concentrations were determined with a competitive histamine ELISA kit (Eagle Biosciences, Nashua, NH). Serum endotoxin levels were measured using an endpoint fluorescent assay (Lonza, Walkersville, MD).

ELISA was used to determine intestinal-type fatty acid-binding protein (FABP2) levels in serum samples. Briefly, black ELISA plates (Thermo Labsystems, Franklin, MA) were coated overnight with an anti-FABP-2 antibody (R&D Systems, Minneapolis, MN) and non-specific binding was blocked by overnight incubation with blocking buffer (carbonate buffer with 3% non-fat dry milk, 0.1% Kathon). Serum samples were used in a 1:4 dilution and rat recombinant FABP-2 (Cayman Chemical Company, Ann Arbor, MI) served as standard. Plates were washed on an automatic 96-well plate washer. Captured antigen was detected using a biotinylated capture antibody (R&D System), a streptavidin alkaline phosphatase conjugate (Promega, Madison, WI), and the fluorescent alkaline phosphatase substrate AttoPhos® (Roche, Indianapolis, IN). All ELISA assays were read on a FluoroCount fluorescent plate reader (Packard Instrument Company, Meriden, CT).

Statistical analysis

Statistical significance was determined by unpaired Student's t test or one-way ANOVA, as appropriate, using Prism software. Differences were considered significant when $p < 0.05$. All error bars represent the SEM.

Results

DHCA is associated with ischemia/reperfusion injury of the intestine

Clinical signs of systemic inflammation almost immediately follow reperfusion after DHCA. To determine whether intestinal damage is a primary event in this setting and therefore potentially causal to systemic responses, we examined the macro- and microscopic aspects of different gut regions in the early period after DHCA.

Two hours after separation from CPB, the most striking finding was a massive hemorrhagic infiltration of the terminal 5 cm of ileum, which was present in all animals exposed to DHCA (Figure 1A). Consistently, histology revealed that the most profound injury was in the ileal specimen, with blunting of intestinal villi as well as extensive hemorrhage and necrosis of the villus tip area. This was associated with a mixed inflammatory infiltrate

(Figure 1D, also see Figure 7A,B). Although overall tissue destruction was less pronounced in caecal and colonic sections, here too, we found increased inflammatory infiltration (Figure 1E). FABP2 is a small cytoplasmic protein that readily leaks out of cells damaged by ischemia. We observed a significant increase in serum intestinal-type FABP (FABP2) in DHCA animals, further indicating extensive I/R injury (Figure 2A).

Both renal and pulmonary dysfunction is common following DHCA; however, histological evidence of early organ injury was less impressive in these tissues, as determined by organ injury scores. In lung sections, some perivascular edema and vascular congestion was observed, but these changes were not clearly attributable to DHCA treatment and occurred also in sham-treated (albeit pressure-ventilated) animals. Similarly, histological evaluation of kidneys revealed focal and group necrosis in some animals after DHCA but did not translate into a significantly increased kidney organ injury score (Supplemental Figure 1). Therefore, our histological findings support the notion that DHCA is primarily associated with characteristic I/R injury of distal portions of the gut.

Since our previous work showed that the intestinal mucosa is particularly susceptible to perfusion abnormalities and resultant tissue hypoxia, we next sought to further define the microenvironmental setting of intestinal injury following DHCA. In contrast to controls (Figure 2D,F), we observed vivid immunolocalization of the hypoxia marker Hypoxyprobe[®] exclusively in the epithelial cell layer of both ileal (Figure 2E) and colonic samples (Figure 2G) from DHCA rats, identifying the intestinal mucosa as a primary target of tissue hypoxia. Furthermore, such mucosal hypoxia was associated with the induction of a number of oxygen-sensitive genes from both inflammatory and hypoxia adaptive pathways (Figure 2B). TNF- α and a group of hypoxia-inducible factor-1- dependent genes, including CD73, multidrug resistance gene-1 (MDR1), and glucose transporter-3 (Glut3), were significantly increased in DHCA vs. sham-treated animals.

Another consequence of tissue malperfusion is oxidative stress, which can be assessed by measuring products of lipid peroxidation. The lipid peroxidation product MDA was significantly increased in samples from all analyzed intestinal segments from DHCA vs. sham rats (Figure 2C). These findings collectively indicate that intestinal injury occurs in a microenvironment characterized by tissue hypoxia and oxidative stress.

A major manifestation of intestinal injury is breakdown of the epithelial barrier. This was assessed in Ussing chambers, which allowed the ex-vivo determination of site-specific intestinal barrier function. Using FITC-labeled dextran as a tracer substance, a dramatic disruption of ileal and, to a lesser extent, colonic barrier integrity was confirmed in DHCA animals (Figure 3A). Such a breach of the intestinal barrier was pathophysiologically relevant and was associated with a significant increase in serum endotoxin levels at the completion of the experiment indicating relevant bacterial translocation (Figure 3B). Together, these findings establish the presence of a functionally relevant I/R-type injury of the intestinal barrier in animals undergoing DHCA.

Intestinal I/R injury is associated with local mast cell activation

Since MCs have previously been implicated in intestinal I/R injury^{11,18,19}, we sought to examine the contribution of these cells in our model. As shown in Figure 4A and B, intestinal MCs lacked signs of activation in sham-treated animals and were predominantly seen in the basal regions of the mucosa. Following DHCA however, a significant portion of intestinal MCs was degranulated as a manifestation of their activation (Figure 4C–E). This was paralleled by an overall increase of MC numbers (Supplemental Figure 2) and an enhanced presence of MCs in the apical regions of the mucosa (Figure 4 C,D). In the dependent mesenteries, we found a large number of MCs in close contact with vascular structures (Figure 4F). In DHCA treated animals, mesenteric MCs were degranulating in $77\% \pm 14\%$ (Figure 4G) vs. $15\% \pm 1\%$ in sham cases (Figure 4F) indicating significant MC activation in this compartment.

Intriguingly, the observed MC hyperplasia after DHCA was consistent with increased carboxypeptidase A3 (CPA3)-positive MCs within the intestinal mucosa (Figure 5B). Because CPA3 expression is generally thought to be associated with connective-type MCs rather than mucosal type MCs, we aimed to further characterize the MC phenotype in colonic mucosal scrapings by mRNA profiling. Following DHCA, we found a significant increase in CPA3 and MC protease-5 and -6 signal, indicative of increased connective type MCs. MC protease-1 and -7 mRNA levels, which are typically associated with mucosal MCs (Figure 5C) however remained stable. Taken together, these data suggest that splanchnic MC activation after DHCA is associated with changes in MC distribution and phenotype.

Mast cell stabilization leads to decreased intestinal I/R injury and an abrogated systemic inflammatory response

We next investigated the functional relevance of MC activation in this model. Systemic cromolyn sodium (CS) is an FDA-approved MC stabilizer for the symptomatic treatment of mastocytosis. To test the efficacy of CS in blocking MC degranulation, immunofluorescence staining for tryptase was performed in our model. Sham-treated animals displayed a punctate staining pattern within the lamina propria when probed with antibody to tryptase, consistent with tryptase contained within intestinal MCs (Figure 6A). DHCA animals treated with vehicle demonstrated diffuse release of tryptase into the villus stroma, which coincided with the loss of epithelial cell sheaths in the villus tip region (Figure 6B). In contrast, DHCA rats treated with CS exhibited the same tryptase staining pattern seen in sham animals, but the increased tryptase signal suggested, as shown before, that substantial MC hyperplasia had occurred (Figure 6C).

Correspondingly, we observed dramatic increases in serum levels of a further MC product, histamine, immediately following reperfusion (Figure 6D) and a continued rise during the recovery period in DHCA vs. sham animals. However, this response was significantly blunted when animals were pretreated with CS before DHCA. Animals subjected to hypothermic CPB without circulatory arrest (DHCPB) did not show significant histamine release, suggesting that neither hypothermia nor blood exposure to extracorporeal circulation were major factors in MC activation.

Having established the efficacy of CS in suppressing MC activation, we examined the effect of CS on the macroscopic (Figure 6E compared to Figure 1A) and microscopic (Figure 6F,G compared to 1D,E) appearance of the gut in DHCA-treated animals. CS treatment resulted in an almost complete preservation of the intestinal barrier. This was demonstrated in a pathophysiologically relevant manner by significantly lower LPS serum levels in CS- vs. vehicle treated animals after DHCA (Figure 6H). Furthermore, measurements of FITC dextran flux in Ussing chambers showed that the main site of injury, the ileal barrier, was significantly better maintained after DHCA in CS-treated rats (DHCA-vehicle vs. DHCA-CS: 0.01147 ± 0.00061 mg/min vs. 0.0056 ± 0.00105 mg/min, $p = 0.05$). A corresponding trend was observed in colon samples (data not shown).

As indicated above, MCs contain large stores of preformed proinflammatory cytokines, which may profoundly affect local and systemic inflammatory responses. In vehicle-treated animals, a significant number of neutrophils were observed in the intestinal inflammatory infiltrate after DHCA culminating in crypt abscess-like lesions at the villus base (Figure 7A, B white arrow). MC stabilization with CS, however, resulted in abrogation of neutrophil influx documented both histologically (data not shown) and via MPO measurements (Figure 7C).

Furthermore, MC stabilization resulted in significant alteration of systemic inflammatory responses. A number of proinflammatory cytokines were significantly increased in the serum of DHCA-treated animals 2 hrs. after separation from CPB (Figure 8). However, in DHCA animals pretreated with CS, these responses were significantly blunted for TNF- α , MCP-1, RANTES, IL-1 β , and the IL-8 homologue GRO/KC and showed a trend toward reduction in IL-10. In addition, serum MIP-1 α levels were significantly lower compared to vehicle-treated animals after DHCA. Serum levels of IFN γ (Figure 8B), and IL-4 and IL-12 (not shown) did not reveal a clear trend following DHCA with or without CS. These data thus support that MC activation plays a pivotal role in the orchestration of local and systemic early inflammatory responses.

Discussion

Both the nature and consequences of intestinal injury following major cardiovascular surgery have been poorly defined. In a rat model of DHCA, we provide primary evidence of a prominent intestinal I/R-type injury characterized by extensive epithelial hypoxia, oxidative stress and increased intestinal leakage. In this setting, we demonstrate not only significant MC hyperplasia and activation within the intestinal mucosa, but we also establish a role for MC in local injury and systemic inflammatory responses.

The anoxic lumen of the gut and its dependence on a complex vascular bed provide an anatomical setting in which relatively small changes in blood flow may lead to a significant disruption of blood supply particularly in the villus tip area.²⁰ Various studies suggest that during CPB, changes in microvascular perfusion and intestinal metabolic demands occur.^{21, 22} Our data documents that mucosal hypoxia and oxidative stress constitute defining microenvironmental factors that lead to epithelial injury. Interestingly, in our model, such injury had a reproducible predilection for the terminal ileum – a watershed area of the

superior and inferior mesenteric blood supply and preferred site of injury in both Crohn's disease and necrotizing enterocolitis. In all these conditions, hypoxia/ischemia is thought to play at least a contributory role^{4, 23} potentially indicating a broader role of oxygen supply in the development of intestinal inflammation.

As an important consequence of intestinal injury in cardiac surgery, much interest has focused on bacterial translocation and resultant systemic inflammatory responses (reviewed in 7). However, more recently this "gut-origin" hypothesis of systemic inflammation has been expanded to account for the immense proinflammatory potential of the gut resident immune system.²⁴ Two major notions suggested that intestinal MC play a previously unappreciated role in this process.

First, our data characterizes DHCA-related intestinal damage as an I/R type injury. Various reports underline the importance of MC activation in this setting, as both MC deficiency^{10,11} and MC stabilization¹⁹ significantly reduce local inflammation and injury scores in mouse models of mesenteric I/R.

Second, a systemic response, characterized by hemodynamic instability and coagulation abnormalities, often occurs immediately after DHCA, implying a role of preformed effectors. Consistently, our studies focused on the early responses to DHCA and revealed that activation of MCs, which are submucosal immune surveillance cells, contributes significantly to DHCA-induced breakdown of the intestinal barrier. MCs are powerful secretory cells capable of releasing a panoply of preformed and de novo synthesized mediators. As the major source of preformed proteases, mediators and various cytokines (e.g. TNF- α ²⁵), MCs can exert powerful local and systemic effects. As such, MC-tryptase²⁶, which is prominently released into the intestinal tissue in our model, as well as MC-protease⁴²⁷ have been implicated in the breakdown of tight junctions in various mucosal epithelia including the gut. While we were unable to assay serum tryptase levels, possibly because it binds to circuit components (data not shown), serum levels of histamine – another but less specific MC product – significantly increased immediately after reperfusion. This concurs with the proposed role of MCs in the early response to DHCA and suggests that, in the series of events, MC activation precedes epithelial injury (FABP2 release) and breach of intestinal barrier (LPS serum levels).

Interestingly, we found that MC activation was associated with a rapid increase in the intestinal MC population. Further characterization of this MC hyperplasia was performed using mRNA profiling of MC-specific proteases, which may not correspond to overall MC numbers,²⁸ but conveys a representative picture of the prevailing MC phenotype.²⁸ Most notably, we observed a significant increase of CPA3 mRNA levels, which was confirmed by increased immunohistochemical detection of CPA3 positive cells immediately below the mucosa. Further validation was obtained to some extent by the concomitant increase of MCP-5 mRNA levels, as their expression is linked and CPA3 knockout mice are deficient in MCP-5,²⁹ and vice versa.³⁰ In contrast, MCP-1 remained unaltered after DHCA, indicating that MC expansion is not associated with increase in cells bearing markers typical for the mucosal MC population. Although CPA3 was previously thought to be absent in rodent mucosal MCs,³¹ but such exclusivity has been questioned, at least in the context of airway

inflammation.^{28,32} The postulated role of CPA3 in MC ontogeny²⁹ leads to the speculation that intestinal I/R may augment the numbers of immature MCs within the mucosa in our model. Ultrastructural studies have revealed that MC progenitors are found not only in peripheral blood but also in tissue³³ where they may form a cell pool that can be rapidly expanded, as seen in our series. As will be discussed below in more detail, MCs play an important role in defense of local infections;³⁴⁻³⁶ the observed prompt increase in MC numbers could therefore represent an attempt to limit bacterial spread associated with disruption of the intestinal barrier.

The implications of intestinal MC activation in the setting of DHCA were revealed using cromolyn sodium (CS), which had a profound effect on macro- and micropathological and functional features of intestinal injury. As described above different MC proteases may play an immediate role in barrier disruption following I/R. In addition, MCs have the unique ability to store and rapidly release preformed TNF- α ,²⁵ thus effectively regulating neutrophil influx.³⁴ In our model, both serum TNF- α levels and local myeloperoxidase levels were significantly reduced with CS treatment. Modification of neutrophil recruitment thus provides an additional explanation for the dramatic differences in tissue disruption between treatment groups and is consistent with other studies of intestinal I/R injury.^{10,37}

MCs are pivotal initiators of immune responses to infection. However, mounting evidence suggests a Janus-headed role of MCs in which local activation limits bacterial infections whereas excessive MC activation leads to clinical deterioration.^{12,36,38,39} In the context of cardiac surgery, a still unclear interplay of a number of factors may lead to such an exaggerated MC response. Here, microenvironmental conditions such as hypoxia and oxidative stress, may well account for the observed local MC activation.^{18,40} On the other hand, complement components 3a and 5a – both well established MC activators⁴⁴ – regularly increase following CPB.^{41,42} These factors or other inflammatory signals associated with cardiac surgery may increase general MC responsiveness and promote an inflammatory cascade that originates in submucosal MCs but is amplified to systemic magnitude by the vast mesenteric MC population. While more work is needed to clarify this complex interplay of inflammatory stimuli, our data, using cromolyn sodium, strongly suggests that MC activation is a key event shaping this inflammatory response.

As a further sign that MC products do gain access into the circulation, we observed a very early and cromolyn-modifiable rise in a number of serum-cytokines, indicating release from preformed stores and as such, confirming results by McILwain et al⁴¹ in a neonatal extracorporeal membrane oxygenation (ECMO) model. MCs have been identified as the major source of preformed TNF- α .^{25,36,39,41} Additional cytokines, that were elevated after DHCA, such as IL-1 β ,⁴³ monocyte chemotactic protein 1 (MCP-1), IL-8, RANTES, and macrophage inflammatory protein 1 α (MIP-1 α),⁴⁴ have also been described as MC products and are known to exert profound influences on the progression of a number of inflammatory pathways.

In conclusion, we have characterized intestinal I/R injury as a primary event following DHCA and provide evidence for the previously poorly appreciated role of MCs in the initiation of local and systemic inflammation after DHCA. The successful preclinical trial of

a safe and efficient MC stabilizer furthermore provides a novel therapeutic opportunity to modify such potentially harmful inflammatory processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Intestinal injury in a rat model of deep hypothermic circulatory arrest (DHCA)
(A) Representative photograph of harvested distal portion of the gut displays the characteristic hemorrhagic infiltration of the terminal ileum after DHCA (asterisk). In hematoxylin and eosin staining, sham-treated animals present a morphologically unremarkable histology of ileum **(B)** and colon **(C)**. After DHCA, significant mucosal destruction predominantly of the apical villus is seen. In accordance with the macroscopic presentation, such injury is more extensive in the ileum and contains a strong hemorrhagic component **(D)**. In the colon, mixed inflammatory infiltrate (arrow) leads to increased lamina propria cellularity **(E)**. (Magnification x 200)

Figure 2. Epithelial hypoxia and oxidative stress of the gut wall following deep hypothermic circulatory arrest (DHCA)

(A) Intestinal-type fatty acid binding protein was assessed in the serum of rats exposed to sham conditions or deep hypothermic cardiopulmonary bypass (DHCPB) or deep hypothermic circulatory arrest (DHCA). Serum samples were obtained after induction of anesthesia (Induction), 5 minutes after reperfusion (Reperfusion), or 2 hours after separation from bypass (Recovery). (B) Quantitative PCR of hypoxia-responsive gene transcription of TNF- α , CD73, glucose transporter-3 (Glut3), and the multiple drug resistance gene-1 (MDR1) was performed in colonic scrapings. (C) Oxidative stress was evaluated by measuring malondialdehyde (MDA) in whole tissue homogenates from ileum, caecum, and colon. Animals were pretreated with Hypoxyprobe®, a marker of cellular hypoxia (red color, blue is DAPI nuclear counterstain). In sham-treated animals, sections from the ileum (D) and colon (F) reveal only a faint signal in the tip of colonic villi. In contrast, following DHCA, retention of Hypoxyprobe® is dramatically increased (E) in ileum and (G) in colon (Magnification x 200). * p 0.05 vs. sham (n=3-4 per group).

Figure 3. Marked changes in intestinal barrier function following deep hypothermic circulatory arrest (DHCA)

(A) To determine site-specific barrier abnormalities, specimens from ileum and colon were mounted in Ussing chambers and the flux of FITC-labeled dextran was measured. (B) Serum endotoxin was measured in sham-treated animals and animals undergoing DHCA or deep hypothermic cardiopulmonary bypass (DHCPB). Samples were obtained after induction of anesthesia (Induction), 5 minutes after reperfusion (Reperfusion), or 2 hours after separation from bypass (Recovery). * $p < 0.05$ vs. sham ($n=6$ per group, $n=3$ for DHCPB).

Figure 4. Intestinal mast cell activation following deep hypothermic circulatory arrest (DHCA) Colonic sections were stained with toluidine blue. In sham-treated animals, well-defined mast cells are seen predominantly in the lamina propria immediately underlying the colonic crypts (black arrows, [A], Magnification x 200; [B] x 400). Following DHCA, increasing numbers of mast cells appear in the villus tip area and show signs of degranulation ([C] x 200; [D,E] x 400). In mesenteric windows, a large number of mast cells are in close proximity to vascular structures (sham treatment [F]). Following DHCA, a significant portion of mesenteric mast cells shows signs of degranulation (G).

Figure 5. Intestinal mast cell hyperplasia after deep hypothermic circulatory arrest (DHCA) and altered mast cell protease expression

Immunofluorescence staining was performed using the epithelial marker E-cadherin (red fluorescence) and the mast cell-specific carboxypeptidase A3 (CPA3, green fluorescence; Magnification $\times 400$). **(A)** CPA3-positive mast cells are seen in submucosal layers in sham-treated animals. **(B)** Following DHCA, CPA3 is increasingly detected within the mucosa.

(C) Quantitative PCR from colonic epithelial enriched fractions using specific primers for rat mast cell protease (MCP)-1, 5, 6, 7, and CPA3. β Actin was used to normalize expression.

*p < 0.05 vs. sham; DHCPB: deep hypothermic cardiopulmonary bypass.

Figure 6. Effects of cromolyn sodium treatment on intestinal barrier integrity after deep hypothermic circulatory arrest (DHCA)

(A) Immunohistochemistry of small intestinal sections reveals circumscribed staining of mast cell tryptase (white arrows; Cy3-tryptase, DAPI nuclear counterstain). (B) Following DHCA, tryptase is released into the tissue and is associated with epithelial injury at the villus tip (arrowheads). (C) When DHCA animals were pretreated with cromolyn, the punctate staining pattern seen in sham-treated animals is maintained, but overall numbers of mast cells appear augmented (white arrows). (D) Serum histamine levels were determined by competitive ELISA. Samples were obtained after induction of anesthesia (Induction), 5 minutes after reperfusion (Reperfusion), or 2 hours after separation from bypass (Recovery). (E) Representative photograph of the distal gut portion in a cromolyn-treated rat after DHCA. (Asterisk indicates ileocaecal region; compare to Figure 1A.) Hematoxylin and eosin staining of ileum (F) and colon (G) documents largely maintained wall architecture following DHCA in animals pretreated with cromolyn (compare to Figure 1D,E). (H) Serum LPS was determined from samples obtained 2 hours after separation from cardiopulmonary

bypass. Abbreviations: Sham/DHCA Vh – pretreatment with saline (vehicle); Sham/DHCA CS – pretreatment with cromolyn sodium; DHCPB Vh – deep hypothermic cardiopulmonary bypass, pretreatment with saline. *p 0.05 vs. Sham Vh; # p 0.05 vs. DHCA Vh (n=6 per group, n=3 for DHCPB).

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Figure 7. Effect of mast cells on intestinal neutrophil infiltration after deep hypothermic circulatory arrest (DHCA)

(A) Hematoxylin and eosin staining of the ileum shows the mixed inflammatory infiltration of the lamina propria after DHCA (Magnification x 400). (B) The higher magnification focuses on a crypt abscess-like lesion and reveals a significant neutrophil component (black arrows, Magnification x 800). (C) Myeloperoxidase assay from tissue homogenates. Ileal and colonic samples harvested 2 hours after separation from cardiopulmonary bypass. Abbreviations: Sham/DHCA Vh – pretreatment with saline (vehicle); Sham/DHCA CS – pretreatment with cromolyn sodium; DHCPB Vh – deep hypothermic cardiopulmonary bypass, pretreatment with saline. *p 0.05 vs. Sham Vh; # p 0.05 vs. DHCA Vh (n=6 per group, n=3 for DHCPB).

Figure 8. Effect of mast cell stabilization on systemic cytokine response after deep hypothermic circulatory arrest (DHCA)

Multiplex cytokine ELISA from serum samples obtained 2 hours after separation from bypass. *p 0.05 vs. Sham Vh; # p 0.05 vs. DHCA Vh (n=6 per group, n=3 for DHCPB). Abbreviations: Sham/DHCA Vh – pretreatment with saline (vehicle); Sham/DHCA CS – pretreatment with cromolyn sodium; DHCPB Vh – deep hypothermic cardiopulmonary bypass, pretreatment with saline. *p 0.05 vs. Sham Vh; # p 0.05 vs. DHCA Vh (n=6 per group, n=3 for DHCPB).