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Smooth muscle actin as a novel serologic marker of severe intestinal damage in rat intestinal ischemia–reperfusion and human necrotising enterocolitis

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ABSTRACT

Background: Despite emergence of markers of intestinal mucosal damage such as intestinal fatty-acid binding protein (i-FABP), there are no specific markers of damage extending into the muscle layers. We hypothesized that smooth muscle actin (SMA) released from the intestinal muscularis would be detectable in plasma after severe intestinal injury.

Materials and methods: Serial blood samples were collected from rats ($n = 10$) undergoing intestinal ischemia–reperfusion injury (IRI) and controls ($n = 5$). Additionally, admission and/or preoperative plasma samples were collected from twelve neonates with necrotizing enterocolitis (NEC), and five age- and weight-matched controls. Plasma ileal fatty-acid binding protein (rat) or i-FABP (human) were measured by enzyme-linked immunosorbent assay, and plasma SMA was detected by western blotting.

Results: Plasma ileal fatty-acid binding protein was low in both the control group and IRI at baseline, but became rapidly elevated in the IRI group even during ischemia. SMA was detected in reperfusion plasma samples of all IRI rats, but in none of the control samples. Plasma i-FABP was higher in infants with NEC than age- and weight-matched controls. Although i-FABP was higher in infants with severe surgical disease compared with focal disease, there was no difference between the operative and nonoperative groups. SMA was detected in the plasma of all four neonates with severe surgical NEC, but not in those with focal disease or those who were successfully conservatively managed.

Conclusions: SMA is detectable in plasma after severe intestinal injury and maybe a clinically useful maker of intestinal muscle damage.

1. Introduction

The diagnosis of intestinal necrosis in conditions such as acute mesenteric ischemia (AMI) [1] and necrotizing enterocolitis (NEC) [2] remains a significant challenge to clinicians. While there is consensus that the presence of transmural intestinal necrosis necessitates surgery to remove any nonviable intestine, [1,2] confirming either the presence or absence of necrotic

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intestine is frequently difficult. Current practice is based on a combination of clinical, radiological, and serologic information, as there is no single test other than exploratory laparotomy that is sufficiently accurate to be considered diagnostic. As both AMI [3] and NEC [4] are associated with high mortality rates, and early diagnosis and treatment may improve patient outcome [4], rapid diagnostic tests with high sensitivity and specificity maybe beneficial. Analogous to the use of serologic markers of cardiac or liver damage, plasma or urinary markers of intestinal ischemia and necrosis could potentially be useful in guiding treatment decisions and in particular the need for surgery. However, recent systematic reviews suggest that no individual serologic marker can be considered diagnostic for either AMI [5] or NEC [6] at present. One potential explanation for this is that many of the proposed serologic or urinary markers originate from enterocytes. Elevated levels are therefore suggestive of enterocyte (i.e., gastrointestinal mucosal) damage rather than damage extending into the muscle layers. For example, intestinal fatty-acid binding protein (i-FABP), a major enterocyte protein, is elevated in plasma and urine in patients with AMI [7–9] and with NEC [9,10], human [11]. However, i-FABP is also elevated in patients with milder degrees of intestinal damage, such as that occurring during sepsis [12,13], myeloablative conditioning [14], and nongastrointestinal surgery [15,16]. Thus elevated i-FABP, although potentially useful as a diagnostic test, cannot currently be regarded as an indication for surgery in an infant with NEC [17].

Given that transmural intestinal necrosis involves the intestinal muscle layers and the mucosa, we hypothesized that alpha smooth muscle actin (SMA), a component of smooth muscle would be released into the circulation in severe intestinal damage. The aims of this study were to (A) examine the pattern of i-FABP and SMA release in a rat model of intestinal ischemia–reperfusion injury (IRI) and (B) determine whether SMA is detectable in plasma of human infants with NEC.

2. Materials and methods

2.1. Rat intestinal IRI model

All animal experiments were conducted with approval from the United Kingdom Home Office. Adult male Sprague–Dawley rats weighing between 200 and 250 g were anesthetized using isoflurane at a concentration between 1% and 3% mixed with oxygen. Normothermia (rectal temperature 36°C–38°C) was maintained with a heating lamp and blanket. The caudal artery was cannulated to allow for plasma sampling. The abdomen was opened with midline laparotomy, the superior mesenteric artery identified, and the vascular pedicle dissected free. Intestinal ischemia was induced by the placement of a vascular microclip across the origin of the superior mesenteric artery. The abdomen was then closed and the clip was left in place for 60 min. After 60 min, the abdomen was reopened and the clip removed allowing reperfusion to commence. Reperfusion was confirmed by visualization of pulses in the mesenteric arterial arcades. The abdomen was subsequently closed for a final time, and reperfusion continued for upto 150 min. The experiment was terminated when either the animal died or was euthanized at 210 min. Plasma sampling (200μL per sample) occurred at the onset of ischemia (t = 0 min) and at 30, 55, 65, 75, 90, 120, 150, 180, and 210 min after the onset of ischemia. Animals in the control group underwent a sham procedure, which was identical in every way except that no vascular clamp was placed across the superior mesenteric artery.

2.2. Human NEC plasma samples

In a pilot study, two groups of neonates who were treated in the Neonatal Intensive Care Unit (NICU) at Great Ormond Street Hospital between 2002 and 2003 were enrolled in an ethically approved prospective observational cohort study. The study group consisted of neonates with definite NEC (Bell Stage II or III). A control group consisted of corrected gestational age- and weight-matched patients who were admitted to NICU without NEC, sepsis or septic shock, systemic inflammatory response syndrome, or an inborn error of metabolism. For the purposes of this study, infants were classified as either having NEC requiring surgery (operative NEC) or NEC that was treated medically without surgical intervention (nonoperative NEC). In infants with operative NEC, blood samples taken preoperatively were analyzed and compared with operative findings. In infants with nonoperative NEC and in control infants blood samples taken on admission to our unit were analyzed. Whole blood was collected in EDTA specimen tubes, centrifuged at 3000 rpm for 3 min, the plasma separated and stored at −80°C until analysis. Clinical and demographic data were collected from all patients, including intraoperative findings, surgical procedures performed and clinical outcome at the point of discharge. The extent of intestinal disease encountered in infants with operative NEC was classified as focal when a single segment was affected, multifocal if two or more segments were affected, and panintestinal if >50% of the small and large bowel were affected [18].

2.3. Measurement of rat il-FABP and human i-FABP

The concentration of ileal fatty-acid binding protein (il-FABP), a similar protein to i-FABP with a more ileal specific distribution, in rat plasma was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) for murine il-FABP that cross-reacts with rat il-FABP (HBT, The Netherlands) according to the manufacturer's protocol. Briefly, plasma was diluted 1:10 and incubated in precoated ELISA plates. The samples were discarded, the ELISA plate washed three times with phosphate-buffered saline, and a biotinylated secondary antibody added for 1 h. After a further three wash steps, the wells were incubated with streptavidin peroxidase for 1 h. After a final three wash steps, 3,3′,5,5′-Tetramethlybenzidine was added for approximately 30 min in the dark, before the reaction was stopped by the addition of citric acid. The plate was read at 450 nm and il-FABP concentration was quantified by comparison with a set of predetermined standards.

Human plasma i-FABP concentrations were also measured using a commercially available ELISA kit (HBT) using an otherwise identical technique to the rat il-FABP ELISA.