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Prevention of intestinal ischemia-reperfusion injury in humanized mice

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Abstract

Background—Ischemia-reperfusion injury (I/R) is an inflammatory response to hypoxia that is dependent on antibodies. We have previously shown that the N2 peptide blocks binding of auto-reactive antibody to ischemic tissue and reduces I/R injury in rodents. Whether these same antibodies exist and target the same antigen in humans is unknown. In this study, we create a novel “humanized” mice model of intestinal I/R and test the efficacy of N2.

Methods—Humanized mice were generated by transplanting human lymphocytes into immunodeficient mice. Human T and B cell engraftment in the spleen and peritoneum was confirmed using flow cytometry, and circulating human antibody in mouse serum measured with ELISA. Injury in the small intestine and lung after intestinal I/R was measured in the presence or absence of N2. Immunohistochemistry was used to assess for human antibody deposition in small intestine.

Results—Humanized mice engrafted with functional CD20+ B cells that generated high circulating serum levels of human antibody. N2 treatment significantly reduced intestinal injury severity scores (ISS) after I/R (control: 28 ± 1.5 , N2: 9.1 ± 3.4 ; $p < 0.05$). N2 also attenuated remote lung inflammation after I/R (control: 28 ± 4 , N2: 5.4 ± 1.3 ; $p < 0.05$). Protection from I/R injury correlated with blockade of human antibody deposition on small intestine.

Conclusions—N2 is an effective therapy for I/R injury in the presence of human immunity, supporting a conserved target of inflammatory attack in human reperfusion injury.

INTRODUCTION

Ischemia-reperfusion (I/R) induces an inflammatory response that causes local tissue damage as well as activation of a systemic inflammatory response syndrome leading to remote tissue injury. I/R is a clinically important phenomenon that occurs during the natural course of therapy for myocardial infarction, trauma, and peripheral arterial embolism.

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I/R injury is dependent on complement, antibodies, and peritoneal B cells (1-4). Our group has previously identified an IgM antibody called CM22 that restores intestinal ischemia/reperfusion injury in antibody deficient mice (5-6). The target of CM22 was identified and found to correspond to a widely-expressed cytoskeletal protein, non-muscle myosin heavy chain II-A (NMHC-IIA) (7).

N2, a peptide corresponding to a conserved C-terminal section of mouse and human NMHC-IIA, binds CM22 *in vitro* (7). N2 blocks IgM antibody deposition and inhibits injury in rodent models of intestinal, skeletal muscle, and myocardial I/R injury, as well as burn injury and hemorrhagic shock (8-13). Thus, NMHC-IIA appears to be a conserved injury/ischemia antigen in multiple rodent tissues. A critical, unanswered question is whether human antibodies that target NMHC-IIA exist, and if so, whether NMHC-IIA is a conserved injury antigen in humans.

To address these questions, we engrafted NOD.SCID.IL2rg knockout mice, which lack endogenous murine NK cells, T, and B cells, with human peripheral blood lymphocytes to generate humanized mice (15-16). Prior studies have shown that these mice stably engraft with human T cells and generate at least some human antibody isotypes (17, 18). We characterize B cell and antibody expression in this model, as well as the phenotype of intestinal I/R injury. We then test the efficacy of N2 against intestinal I/R using this novel humanized mouse model.

METHODS

Generation of humanized PBL-SCID mice

NOD.PkSCID.IL2 receptor gamma chain null mice were purchased from Jackson Laboratories (Bar Harbor, ME). Human lymphocytes were obtained by Ficoll-Paque density centrifugation from peripheral blood obtained from healthy adult, male donors. 3-4 week old male mice were injected with 20-25 million human lymphocytes i.p. Mice were allowed to engraft for 2-4 weeks prior to use in I/R experiments. Engraftment was verified by ELISA analysis of serum samples obtained by retro-orbital bleeding. For examination of the efficacy of N2 treatment, all mice were generated from a single healthy adult male donor to limit variability introduced by use of different lymphocyte donors. Animal experiments were approved by the Institutional Animal Care and Use Committee.

Intestinal ischemia/reperfusion

Mice were anesthetized with pentobarbital. Mice were injected i.v. with either 300 µg N2 peptide or as a control, an equivalent volume of vehicle (PBS) alone. This dose of N2 peptide was chosen based on a previously performed dose-response curve of N2 efficacy in intestinal I/R and burn injury in wild-type C57BL/6 mice (7, 9). Each group contained between 3-5 animals. Subsequently, under aseptic conditions, a laparotomy was performed, and the SMA was isolated and occluded with a microvascular clamp using a dissecting microscope. Ischemia was confirmed by pallor of the small intestine. The abdomen was closed temporarily with non-absorbable suture. Following 40 minutes of ischemia, the abdomen was reopened, and the clip removed to initiate reperfusion. Animals were hydrated

with 300 μ l saline, and the abdomen closed with non-absorbable suture. Animals were maintained on a heating barrier pad throughout reperfusion. Following 3 hours of reperfusion, animals were sacrificed by pentobarbital overdose.

Flow cytometry

Splenocytes were purified by standard methods using mechanical disruption and hypotonic red blood cell lysis. Mice peritoneal cavities were lavaged with 5 ml of sterile PBS. Splenocytes and peritoneal cells were blocked with PBS/10% FCS for 20 minutes on ice and then stained with FITC-anti human CD19 (Becton Dickinson), PE- anti human CD5 (Becton Dickinson), APC-anti-human CD20 (Biolegend), FITC-anti human CD4 (Biolegend), FITC-anti human CD8 (Biolegend), or APC-anti human CD3 as indicated for 30-60 minutes at 4 degrees in PBS/2% FCS. Cells were washed in PBS/2% FCS and fixed in PBS/2% paraformaldehyde. Cells were analyzed using a BD FACSCalibur and analyzed using WINMDI software.

ELISA

Animals were bled retro-orbitally, and serum isolated using microtainer tubes (BD). Nunc MaxiSorp plates were coated with 10 μ g/ml of polyclonal goat anti-human IgM, IgG, or IgA (Southern Biotech) overnight at 4 degrees in carbonate buffer. Plates were washed 5 $\times$ with PBS/0.05% Tween, blocked with 10% fetal calf serum, and serum samples incubated overnight at varying concentrations. Plates were washed in PBS-T, and then incubated in 1:10,000 biotinylated anti-human IgM, IgG, or IgA (Jackson ImmunoResearch), followed by 1:1000 streptavidin-alkaline phosphatase (Sigma), and developed using PNNP. Plates were washed in PBS-T between each incubation. Plates were read at 405 nm using a spectrophotometer.

Pathology and injury scoring

Small intestine was fixed in 10% formalin, and tissues were dehydrated and paraffin embedded by standard methods (Rodent Histopathology Core, HMS). Longitudinal sections of the entire small bowel were H&E stained and scored for pathologic evidence of intestinal injury by the Chiu score in a blinded fashion (19). Lung injury was scored by counting numbers of neutrophils infiltrating the alveoli. Ten random high powered (400 $\times$) fields were scored for each animal in a blinded fashion.

Immunohistochemistry

Paraffin embedded small intestine sections were rehydrated in PBS. Endogenous peroxidases were quenched in 3% hydrogen peroxide in methanol for 20 minutes, and antigen retrieval was performed using citrate buffer (IgM and IgG). Sections were incubated with biotinylated anti-human IgM (1:250, Vector labs) or biotinylated anti-human IgG (1:250, Vector labs) overnight at 4 degrees. Following washing in PBS-tween, IgM and IgG stains were developed using the Elite ABC system per manufacturer's instructions (Vector Labs). Sections were counterstained in hematoxylin, dehydrated, and coverslipped with Permount.

Data analysis

Data analysis was performed using Microsoft Excel. Injury severity scores and lung inflammatory scores are presented as mean values of individual animals, with error bars representing the standard error of the mean. The Mann-Whitney U test was used to compare the two groups, with significance set at a level of $p < 0.05$.

RESULTS

Functional human B lymphocytes engraft in the spleen and peritoneum of humanized mice

Humanized mice were generated by transferring purified human peripheral blood lymphocytes into NOD.PkSCID.IL2 receptor gamma chain knockout mice, which lack a normal mouse adaptive immune system. Humanized mice engrafted with human CD20⁺ B cells in the spleen and peritoneum (Figure 1, mean frequency: 3.5% and 0.73%, respectively). The majority of the CD20⁺ B cells in the peritoneum expressed CD5, a cell surface marker of B1 cells, which are the primary source of natural antibodies. There were CD5^{dim} and CD5^{bright} sub-populations of B cells (Figure 1). Cell surface expression of CD19 was down-regulated compared to normal human peripheral blood B cells, perhaps reflecting a heightened activation state of B cells in the humanized mouse (data not shown). High frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ human T lymphocytes were confirmed, as has been previously demonstrated (data not shown).

Serum was tested for human Ig by ELISA to confirm the functionality of engrafted B cells. All animals expressed high titers of serum IgA, IgG, and IgM (Figure 2). Engraftment of human B cells and human antibody production was highly reliable, with near 100% efficiency. IgM antibody titers in humanized mice are comparable to those found in healthy adult human serum, as previously reported (18).

N2 treatment prevents local and remote intestinal ischemia-reperfusion injury

We tested the impact of N2 peptide on intestinal I/R injury in humanized mice. Humanized mice undergoing laparotomy without mesenteric ischemia showed no histologic evidence of intestinal injury (data not shown). After intestinal I/R injury characteristic histologic changes of reperfusion injury are seen in the intestine of humanized mice such as sub-epithelial edema and villus disruption (Figure 3A). Treatment with N2 peptide blocked intestinal I/R injury (Figure 3A). Injury severity scores was reduced over 3 fold in N2 treated compared to control animals (Figure 3B, $p < 0.05$).

We next examined the influence of N2 on remote lung injury after I/R. Intestinal I/R results in remote lung injury, manifest by neutrophil infiltration and capillary leak (20). Histologic examination demonstrated polymorphonuclear leukocyte (PMN) infiltration and edema within lung alveoli after intestinal I/R in humanized mice (Figure 4A). N2 treatment reduced the lung inflammatory infiltrate by over 5-fold compared to control animals (Figure 4B, $p < 0.05$).

Human antibody titers for all three isotypes were assessed in control and N2 treated groups and found to be no different (Figure 2). Therefore, reduced I/R injury in the N2 group was not due to baseline differences in levels of human antibody. In summary, these data demonstrate N2 treatment protects humanized mice from the local and remote consequences of intestinal I/R.

N2 treatment prevents I/R induced intestinal deposition of human IgG

We next tested whether intestinal I/R injury in humanized mice correlates with binding of human antibody to mouse intestinal epithelium. We were unable to detect significant levels of human IgM deposition on humanized mouse small intestine using immunohistochemistry (IHC). As engrafted human B cells in these mice demonstrate signs of heightened activation, we postulated that Ig class switching of the auto-reactive antibody producing B cells may have occurred. We therefore looked for human IgG deposition in the intestine. Significant human IgG staining in the subepithelial space of the small intestine was seen after I/R (Figure 5). N2 treatment blocked hIgG deposition in the intestine. In addition, in the absence of injury, hIgG staining was only seen within capillaries of the intestine. Thus, protection from I/R injury in humanized mice correlates with blockade of human IgG deposition in the intestine.

DISCUSSION

We describe a novel model of intestinal I/R injury in mice carrying a human adaptive immune system. Treatment with N2 peptide strongly inhibits both the local and remote injury caused by intestinal I/R in this humanized model. I/R injury correlated with human IgG deposition in the intestinal epithelium, which is blocked by N2 administration. This work confirms and extends prior work that demonstrated restoration of skeletal and intestinal I/R injury using human serum, purified antibody, and peripheral blood lymphocytes (14, 18). This study is the first demonstration that N2 peptide blocks I/R injury mediated by human immunity.

Engraftment with human peripheral blood lymphocytes restores both local and remote I/R injury. Therefore, this study indicates that human peripheral blood lymphocytes are a source of pathogenic I/R antibodies. In mice, B cells producing anti-NMHC-IIA antibodies were first cloned from the peritoneum (5, 24). While it remains possible that the primary source of NMHC-IIA I/R B cells are also within the human peritoneum, the knowledge that these cells are present, either through re-circulation or as predecessors, in the periphery will aid future studies in humans. Of interest, a series of clones of human chronic lymphocytic leukemia (CLL) have been found that express antibodies that bind NMHC-IIA and other cell membrane associated antigens exposed during cell stress (25-26). CLL is thought to arise from a CD5+ B1 cell, adding support to the hypothesis that the natural B cell repertoire carries antibodies recognizing NMHC-IIA. Our humanized mouse model may therefore be an useful resource for isolation and cloning of human CD5+ B cells that trigger I/R injury.

Our data indicate that NMHC-IIA is the key target of autologous inflammatory attack during I/R injury. Besides NMHC-IIA, other targets of the I/R inflammatory response have been proposed, such as annexin IV and β_2 -glycoprotein I (21-23). N2 has no activity against

pathogenic antibodies directed against β_2 -glycoprotein I (21). In our model, N2 blocks the majority of injury and antibody deposition, suggesting that NMHC-IIA is the major target of the inflammatory response in intestinal I/R. However, we do not directly demonstrate binding of human antibody to NMHC-IIA, so the possibility remains that N2 has activity against homologous but distinct ischemia antigens.

In contrast to pure mouse models, the anti-NMHC-IIA antibodies bound on intestinal epithelium are of the IgG and not IgM sub-type. An IgM to IgG antibody subtype change, or class switch, is a well characterized, immunologic phenomenon that occurs in response to sustained antigen stimulation. Class switch allows a more rapid, effective “memory” immune response to subsequent encounters with the same antigen (27). Class switching of NMHC-IIA antibodies to IgG may be an artifact of the huPBL-SCID model system. Flow cytometry of engrafted B cells suggest an activated phenotype, perhaps due to stimulation from mouse xeno-antigens. Thus, it is possible that low grade antigen stimulation leads to class switching from IgM to IgG of anti NMHC-IIA antibodies. However, this level of stimulation must be low, as no human IgG or IgM deposition is seen on mouse intestine in the absence of ischemia.

A significant limitation of our humanized model is that while the B cells and antibodies are of human origin, the small intestine and innate immune system are of mouse origin. Thus, this model is not able to determine whether NMHC-IIA is a naturally exposed human ischemia antigen. Development of a xenograft model carrying human target tissue for I/R injury as well as a human immune system may address this issue.

Our results showing N2 efficacy for intestinal ischemia reperfusion injury in a humanized mouse model give promise to future translation of N2 into clinical therapy. We have previously shown that N2 is effective for I/R injury of multiple tissues besides the gut, including skeletal muscle, myocardium, and the skin (7-12). This conservation of N2 efficacy suggests that injury antigens are shared across tissues, again supporting the possibility that N2 will be effective clinically. One drawback of N2 therapy is the likely need to deliver therapy near the time of injury for optimal efficacy. As the ability to intervene clinically often does not appear until much later after the initial ischemic insult, this is a barrier to N2 therapy. One potential solution is to develop antagonists with longer half-lives and affinities; for example, anti-idiotypic, humanized antibodies, which may have a wider therapeutic window.

In conclusion, we show that N2 is an effective therapy against the local and remote consequences of intestinal I/R injury triggered by human antibodies. This study supports further investigation of N2 and other therapeutics designed to block auto-reactive antibody – ischemia antigen interactions for treatment of reperfusion injury.

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Figure 1. Human B lymphocytes engraft in the spleen and peritoneum of humanized mice
Representative flow cytometry density plots of splenocytes and peritoneal lavage stained for human CD20 and human CD5. Circled gate represent CD20⁺CD5⁺ B1 cells.

Figure 2. Circulating human IgA, IgG, and IgM levels in humanized mice
Serial dilutions of mouse serum were tested for indicated antibody isotypes by ELISA.
Mean antibody titers in control and N2 treated groups are indicated.

(A)**No I/R** **I/R + control** **I/R + N2** **Figure 0003**

(B) **Figure 0004****Figure 3. N2 treatment protects humanized mice from intestinal I/R injury**

(A) Representative H&E stained sections of small intestine from humanized mice that underwent laparotomy without I/R, I/R with control treatment, and I/R with N2 treatment. Arrows indicate areas of epithelial villus disruption. (B) Injury severity scores (ISS) of small intestine after I/R and control or N2 treatment. Mean ISS values are presented with error bars representing standard error of the mean. * $p < 0.05$.

(A)

No I/R

I/R + control

I/R + N2

Figure 0005

(B) **Figure 0006****Figure 4. N2 inhibits remote lung injury after intestinal I/R**

(A) Representative H&E stained sections of lung from sham injured, I/R injured and control treated, and I/R injured and N2 treated humanized mice. (B) Mean numbers of PMN per high powered field in lungs after I/R with control or N2 treatment. Error bars show SEM. * $p < 0.05$.

No I/R

I/R + control

I/R + N2

Figure 5. N2 blocks deposition of human IgG on intestine after I/R

Representative small intestinal sections stained for human IgG (brown) from humanized mice subjected to (A) sham injury, (B) intestinal I/R + control treatment, and (C) intestinal I/R + N2.