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Serum osteopontin is a biomarker of severe fibrosis and portal hypertension in human and murine schistosomiasis mansoni

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Abstract

Schistosomiasis is a major cause of fibrosis and portal hypertension. The reason 4–10% of infected subjects develops hepatosplenic schistosomiasis remains unclear. Chronically infected male CBA/J mice reproduce the dichotomic forms of human schistosomiasis. Most mice (80%) develop moderate splenomegaly syndrome (similar to hepatointestinal disease in humans) and 20% present severe hypersplenomegaly syndrome (analogous to human hepatosplenic disease). We demonstrated that the profibrogenic molecule osteopontin discriminates between mice with severe and mild disease and could be a novel morbidity biomarker in murine and human schistosomiasis. Failure to downregulate osteopontin during the chronic phase may explain why hepatosplenic subjects develop severe fibrosis.

Keywords

Osteopontin; Schistosomiasis mansoni; Liver fibrosis; Portal hypertension; Biomarker

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Infection with *Schistosoma mansoni* is a major cause of fibrosis and portal hypertension (Andrade, 2009; Colley et al., 2014; Lambertucci, 2014). It is estimated that 440 million people from 76 countries and territories are infected, with the majority of cases in sub-Saharan Africa (Chitsulo et al., 2000; Colley et al., 2014). Chronic schistosome infection can present as either hepatointestinal or hepatosplenic clinical forms of schistosomiasis (Andrade, 2009; Lambertucci, 2014). Approximately 90–96% of infected individuals will developed a milder, less symptomatic form of the disease known as hepatointestinal schistosomiasis, presenting few liver granulomas in small vessels of the portal tracts, with minimal or absent liver fibrosis and no portal hypertension (Andrade, 2009; Lambertucci, 2014). The more severe form of the disease, hepatosplenic schistosomiasis, develops in 4–10% of infected individuals and is characterized by numerous granulomas formed in medium and large portal tracts around eggs trapped in non-dichotomous portal branches. This can be followed by severe portal fibrosis and vascular proliferation associated with portal hypertension and splenomegaly (Andrade, 2009; Lambertucci, 2014).

The reason why a small percentage of individuals develop severe fibrosis and portal hypertension and most infected persons do not, is still an open question (Burke et al., 2009). Intensity of infection is one factor associated with hepatosplenic disease but not everyone who has a high intensity infection develops hepatosplenism and not everyone with hepatosplenic disease has a high intensity infection (Andrade, 2004, 2009; Burke et al., 2009; Colley et al., 2014; Lambertucci, 2014). Immunologic and genetic factors have also been associated with severe disease in humans (Andrade, 2004, 2009; Caldas et al., 2008; Burke et al., 2009).

Henderson et al. (1993) observed that inbred male CBA/J mice chronically infected with *S. mansoni* (20 weeks p.i.) recapitulate the human disease, presenting two distinct syndromes: 20% of the mice develop hypersplenomegaly syndrome (HSS, with a pathological presentation similar to hepatosplenic patients) and the majority of mice develop moderate splenomegaly syndrome (MSS, similar to hepatointestinal patients). HSS mice present massive splenomegaly (>550 mg or >1.2% body weight); extensive granulomatous inflammation in medium and large portal tracts; severe, diffuse and systematized portal fibrosis; ascites, thymic atrophy, severe anemia, cachexia, splenic congestion, and portal-systemic collateral circulation (Henderson et al., 1993). By contrast, MSS mice develop moderate splenomegaly; liver granulomas appear isolated in the middle of the parenchyma or in small portal tracts with minimal liver fibrosis and an absence of portal hypertension (Henderson et al., 1993). The differences in pathological presentation are not attributable to differences in intensity of infection.

Mice that develop MSS appear to have a more immunoregulatory phenotype (Freeman et al., 1996). These mice have a greater ratio of T regulatory to T activated cells (Watanabe et al., 2009) and produce higher levels of IFN-gamma (Watanabe et al., 2009) and IL10 (Bosshardt et al., 1997) compared with HSS mice. MSS mice also have distinct anti-soluble egg antigen (anti-SEA) regulatory idiotypes networks that contribute to the immunomodulation of pathology, similar to human schistosomiasis (Colley et al., 1986; Montesano et al., 1989, 1999; Henderson et al., 1993). These findings indicate that subjects that develop the mild

form of the disease are able to modulate the immune response to the pathogen and consequently reduce damage and fibrosis (Henderson et al., 1993; Freeman et al., 1996).

Recently the hedgehog pathway and its target gene osteopontin (OPN), major regulators of several types of liver fibrosis (Omenetti et al., 2011; Syn et al., 2011; Nagoshi, 2014), were demonstrated to play an important role in schistosomiasis (Pereira et al., 2013, 2015). Pereira et al. (2013) showed that the Hedgehog pathway is upregulated in human *S. mansoni* infections and that SEA preparations stimulate liver macrophages to produce Hedgehog ligands, which promote alternative activation of macrophages, fibrogenesis and vascular remodeling. SEA also was demonstrated to directly induce host liver bile duct cells to proliferate and produce the profibrogenic molecule OPN (Pereira et al., 2015). Hepatosplenic patients have increased circulating and hepatic OPN levels compared with hepatointestinal patients (Pereira et al., 2015). Serum/plasma and hepatic OPN levels correlate with the degree of liver fibrosis and the level of portal hypertension, suggesting that this molecule could be a novel biomarker for hepatosplenic schistosomiais (Pereira et al., 2015).

To investigate whether OPN could be a biomarker in schistosomiasis which could differentiate severe fibrosis and portal hypertension from minimal fibrosis, we studied serum levels of OPN in the CBA/J model of chronic schistosomiasis and in patients with different clinical presentations of *S. mansoni* infection.

Male CBA/J mice were obtained from The Jackson Laboratory, Bar Harbor, USA and housed in the American Association of Laboratory Animal Sciences accredited animal care facilities of the Centers for Disease Control and Prevention (CDC), Atlanta, USA. They were infected by s.c. injection of 45 cercariae of a Puerto Rican strain of *S. mansoni* maintained in *Biomphalaria glabrata* snails.

After 20 weeks of infection, mice with MSS and HSS presentations were identified based on the weight of the spleen as described (Henderson et al., 1993; Freeman et al., 1996) (HSS > 550 mg) (MSS, $n = 24$; HSS, $n = 25$). Uninfected mice ($n = 17$) and mice infected with single sex worms ($n = 8$; no eggs, no granulomas (SS/NG)) were used as controls. Mice in the acute phase of the infection (8 weeks, $n = 24$) were included for comparison with animals in the chronic phase. Cardiac blood was collected from anesthetized CBA/J mice before euthanasia and sera were stored at −20 °C until analysis.

A subgroup of mice was followed longitudinally by the use of transponders and blood from the same animals was obtained by retroorbital puncture from anesthetized mice at 6, 8,10,12,16 and 20 weeks p.i. (SS/NG, MSS and HSS, $n = 5$/group). The design of the experiment, the experimental infection with *S. mansoni*, sample collection and euthanasia protocols were approved by the Institutional Animal Care and Use Committee of the CDC (Protocol 1222SECMOUB-A2). OPN was quantified in the serum using OPN Quantikine ELISA kit (R&D Systems, USA) according to the manufacturer's protocol.

A total of 87 plasma samples from patients with schistosomiasis (hepatosplenic – severe fibrosis and portal hypertension: $n = 39$; hepatointestinal – liver granulomas but no periportal fibrosis: $n = 27$; uninfected controls: $n = 21$) diagnosed at the Tropical Diseases

Outpatient Clinic of the Federal University of Minas Gerais (UFMG) Hospital, Brazil were included in the study. OPN was quantified in plasma using OPN Quantikine ELISA kit (R&D Systems, USA) according to the manufacturer's protocol. The present study was conducted in accordance with the Declaration of Helsinki (2013) of the World Medical Association and was approved by the Ethics Committee of UFMG (Protocol ETIC 204/06). Informed consent was obtained from participating subjects.

Results are expressed as medians. Comparisons between groups were performed using a Kruskal–Wallis one-way ANOVA and a Mann–Whitney U test. Significance was accepted at the 0.05 level; Bonferroni correction was applied when comparing more than two groups. Two-way ANOVA and receiver operating characteristics (ROC) curve analysis were used when appropriate. All statistical analyses were performed using SPSS Statistics 22 (IBM) and Prism 6 (GraphPad).

OPN levels were significantly elevated in CBA/J mice with patent infections compared with uninfected controls or animals infected only with worms of one sex which did not produce eggs (Fig. 1A). Mice in the acute phase of the infection (8 weeks) and HSS mice also had significantly more OPN in their sera than MSS mice (Fig. 1A). During the acute phase of the infection, mice that eventually developed MSS or HSS produced high levels of OPN (Fig. 1B). However, in the chronic phase of infection, MSS mice reduced OPN expression to basal levels while HSS mice maintained elevated levels of this profibrogenic molecule (Fig. 1B).

ROC curve analysis demonstrated that serum OPN measurement could be a good biomarker to differentiate mice with severe fibrosis and portal hypertension from mice with more moderate disease (area under the curve (AUROC) 0.8431, $P < 0.0001$) (Fig. 2A).

To further investigate the relevance of the findings from the CBA/J model, we performed the ROC curve analysis in humans with different clinical forms of chronic schistosomiasis. We previously demonstrated that circulating OPN was higher in hepatosplenic patients (Pereira et al., 2015). As observed in Fig. 2B, ROC curve analysis of our cohort of patients indicated that circulating OPN was also a good biomarker to separate patients with severe fibrosis and portal hypertension from patients with more moderate symptoms (AUROC 0.8691, $P < 0.0001$).

The results presented here suggest that OPN may be useful as a biomarker of severe disease in persons with chronic *S. mansoni* infections. Nevertheless, this data should be taken with some caution. Our cohort of patients consists of well-characterized patients from a reference center for tropical diseases. It would be important to validate our data in a larger cohort of patients and under field conditions in order to confirm the use of circulating OPN as a biomarker for severe fibrosis and portal hypertension in human schistosomiasis mansoni.

The modulation of OPN expression in the experimental model which was observed in MSS but not HSS mice suggests that this cytokine may play a role in pathogenesis of schistosomiasis fibrosis and portal hypertension. OPN is a profibrogenic molecule involved in the recruitment of macrophages (O'Regan et al., 2001), activation of hepatic stellate cells (HSC) and collagen synthesis (Syn et al., 2011). In schistosomiasis this molecule has an important role in granuloma formation (O'Regan et al., 2001) but the uncontrolled

expression of this molecule can lead to severe fibrosis (Pereira et al., 2015). We observed that mice and humans with mild disease are able to downregulate OPN in the chronic phase, in association with less severe fibrosis and portal hypertension. Another mechanism that could explain the reduced fibrosis in MSS mice is the increased production of IFN gamma (Watanabe et al., 2009). IFN gamma not only inhibits collagen deposition (Czaja et al., 1989) but it also inhibits OPN expression (Murugaiyan et al., 2010). Thus, IFN gamma could interfere with OPN expression and contribute to its modulation, leading to the milder pathological phenotype observed in MSS mice.

OPN could also contribute to the severe disease observed in HSS mice due to its role in differentiation of Th17 T cells (Murugaiyan et al., 2010). The Th17 phenotype is associated with severe schistosomiasis and IL17 directly induces HSC activation and collagen production, thus contributing to fibrogenesis (Shainheit et al., 2011; Larkin et al., 2012; Tan et al., 2013). Failure to downregulate OPN could lead to an exacerbated Th17 response that also culminates with increased inflammation and fibrosis.

In conclusion, our results suggests that OPN could be a useful tool to identify patients with severe fibrosis in schistosomiasis and lead us to speculate that this cytokine plays a role in the pathogenesis of schistosomiasis fibrosis and portal hypertension. Further studies are necessary to confirm this hypothesis.

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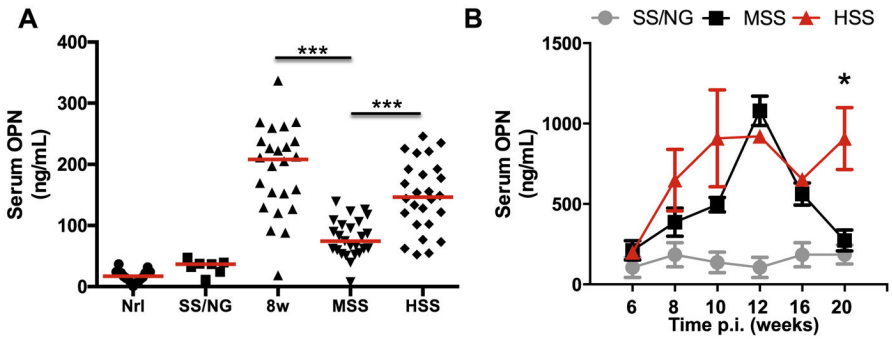

Fig. 1.

Osteopontin is increased in the serum of CBA/J mice with hypersplenomegaly syndrome. (A) Serum osteopontin levels (ng/mL) were measured in uninfected controls (Nrl), mice with single sex worm infections (and hence had no granulomas) (SS/NG), mice in the acute phase of schistosomiasis (8 weeks p.i. (8w)), mice with moderate splenomegaly syndrome (similar to human hepatointestinal schistosomiasis – liver granulomas but no severe fibrosis) and mice with hypersplenomegaly syndrome (similar to human hepatosplenic schistosomiasis – severe fibrosis and portal hypertension). Medians are displayed; $^{***}P < 0.0001$. (B) Longitudinal serum osteopontin levels from SS/NG, moderate splenomegaly syndrome and hypersplenomegaly syndrome mice at different time points of infection. Although during the acute phase moderate splenomegaly syndrome and hypersplenomegaly syndrome have high levels of osteopontin, moderate splenomegaly syndrome mice were able to modulate osteopontin levels to baseline while hypersplenomegaly syndrome mice could not. Two-way ANOVA; $^{*}P < 0.01$.

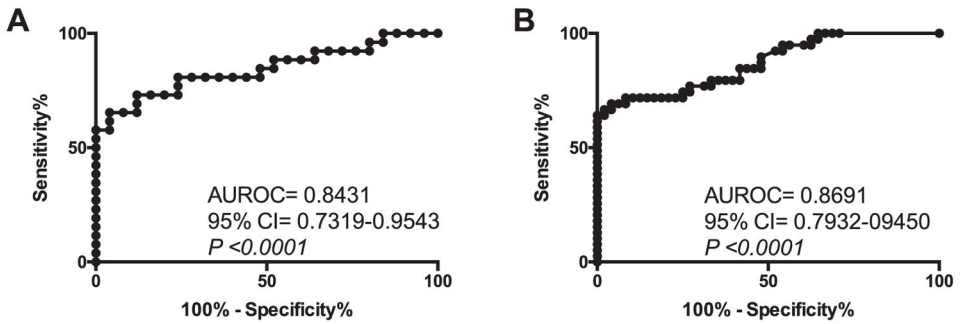

Fig. 2.

Circulating osteopontin is a morbidity biomarker in murine and human schistosomiasis mansoni. Receiver operating characteristics curve analysis of circulating osteopontin levels in murine (A) and human (B) schistosomiasis mansoni. AUROC, area under the ROC curve; CI, confidence interval.