Maternal Infection with *Schistosoma japonicum* Induces a Profibrotic Response in Neonates

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The global burden of schistosomiasis is significant, with fibrosis a major associated morbidity and the primary cause of mortality. We have previously shown that schistosomiasis during pregnancy upregulates proinflammatory cytokines in the cord blood. In this study, we extend these findings to include a large panel of fibrosis-associated markers. We developed a multiplex bead-based assay to measure the levels of 35 proteins associated with fibrosis. Cord blood from 109 neonates born to mothers residing in an area of *Schistosoma japonicum* endemicity was assessed for these molecules. Ten mediators were elevated in the cord blood from schistosome-infected pregnancies, including insulin-like growth factor 1 (IGF-1), tumor growth factor β 1 (TGF- β 1), connective tissue growth factor (CTGF), procollagen I carboxy-terminal propeptide (PICP), amino-telopeptide of type 1 collagen (ICTP), collagen VI, desmosine, matrix metalloproteinase 2 (MMP-2), tissue inhibitor of metalloproteinases 1 (TIMP-1), and TIMP-4. Many of these were also positively correlated with preterm birth (PICP, ICTP, MMP-2, TGF- β 1, desmosine, CTGF, TIMP-1). In addition, birth weight was 168 g lower for infants with detectable levels of CTGF than for those with CTGF levels below the level of detection. Maternal schistosomiasis results in upregulation of fibrosis-associated proteins in the cord blood of the neonate, a subset of which are also associated with adverse birth outcomes. As the first report of fibrosis-associated molecules altered in the newborn of infected mothers, this study has broad implications for the health of the fetus, stretching from gestation to adulthood.

Schistosomiasis currently affects over 200 million people worldwide, including over 40 million women of childbearing age (1), and results in 13 to 15 million disability-adjusted life years (DALYs) lost annually (2). The three primary species responsible for human schistosomiasis are *Schistosoma haematobium*, *Schistosoma mansoni*, and *Schistosoma japonicum*, with *S. japonicum* thought to be the most virulent of the species (3). Fibrosis of the liver is one of the most significant morbidities associated with chronic schistosomiasis and is estimated to occur in up to 20% of people infected with *S. japonicum* (3). In addition to splenomegaly and portal hypertension, which are a direct result of hepatic fibrosis, schistosome-associated fibrosis has also been linked to poor nutritional status and decreased growth (4).

Chronic schistosomiasis is known to elicit production of Th2 cytokines, and this Th2 signature has been associated with the development of fibrosis in both human populations and animal models (5–7). In our earlier studies, stimulation of peripheral blood mononuclear cells (PBMCs) from *S. japonicum*-infected, nonpregnant individuals with schistosome soluble egg antigens (SEA) caused increased production of the Th2 cytokines interleukin 4 (IL-4) and IL-13, which were associated with increased risk of persistent fibrosis 12 months posttreatment (5). A number of other Th2 cytokines, profibrotic growth factors, and mediators of extracellular matrix (ECM) accumulation contribute to the development of fibrosis, including transforming growth factor β 1 (TGF- β 1), thrombin, monocyte chemoattractant protein 1 (MCP-1), and connective tissue growth factor (CTGF) (8). We have previously shown that PBMC production of tissue inhibitor of metalloproteinases 1 (TIMP-1) in response to SEA predicts severe fibrosis in patients for which initial schistosome infection has been

resolved (9), suggesting that these molecules are not just a result of fibrosis but may serve as biomarkers of risk of future fibrosis.

In recent work, we have evaluated the impact of maternal schistosomiasis on fetal development and have shown elevated levels of proinflammatory cytokines in the cord blood of neonates born to infected mothers (10). Vertical transmission of schistosomiasis in humans is not reported; therefore, this proinflammatory response is likely due to antigen trafficking across the placenta (11–17).

Because measures of collagen metabolism may serve as biomarkers of subsequent fibrosis, we evaluated their levels in the cord blood of infants from infected mothers using a multiplexed bead-based assay to quantify a broad spectrum of predictors, mediators, and outcomes of fibrosis. To understand the possible influence of maternal schistosomiasis on expression of these molecules in the neonate, we evaluated cord blood samples from a

Received 23 August 2013 Returned for modification 10 October 2013

Accepted 23 October 2013

Published ahead of print 28 October 2013

Editor: J. A. Appleton

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.01060-13>.

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doi:10.1128/IAI.01060-13

cohort of 109 newborns whose mothers, during pregnancy, resided in a region of the Philippines where *S. japonicum* is endemic.

MATERIALS AND METHODS

Ethical considerations and informed consent. This study was approved by the institutional review boards of Rhode Island Hospital and the Research Institute for Tropical Medicine in the Philippines. All subjects provided informed consent prior to enrollment in the study. Per the guidelines of the Philippine Department of Health, treatment for infections was withheld until after the women had given birth and stopped breastfeeding.

Study site and population. This study was conducted in a region of Leyte, Philippines, in which *S. japonicum* is endemic, HIV prevalence is <0.1%, and there is no malaria. All subjects were pregnant women who presented at a municipal health center for routine prenatal care and were enrolled by trained midwives. Eligibility criteria for this study included the presence of a singleton pregnancy in the second or third trimester, age 18 years or greater, and provision of informed consent. In accordance with the policy of the Philippine Department of Health, no women were treated for schistosomiasis during this study.

Data collection. The collection of all demographic, parasitologic, and birth outcomes data is described in detail in our original publication describing this study population (10). A number of covariates, including gravidity, parity, age, weight, height, smoking status, and socioeconomic status (SES), were determined at the time of enrollment by questionnaire (18). SES was evaluated as a summary score based on answers related to education status, occupational status, and ownership of home, land, and other assets. Geohelminth coinfections (*Ascaris lumbricoides*, hookworm, *Trichuris trichiura*) and schistosome infection were determined from three consecutive stool specimens using the Kato-Katz method. Each stool sample was read in duplicate, and an average number of eggs per gram (epg) was calculated. The intensity of infection for each helminth was determined based on World Health Organization criteria (19). Gestational age was determined at delivery using the modified Dubowitz scoring system or calculated from the date of the mother's last menstrual period (20). Prematurity was defined as delivery of the neonate at <37 weeks of gestation. "Small for gestational age" (SGA) was defined as birth weight below the tenth percentile after adjustment for gestational age and gender based on the Williams curve (21). Cord blood samples were collected at delivery into Vacutainer tubes (Becton, Dickinson, Franklin Lakes, NJ) containing serum separator gel. Serum samples were aliquoted and stored at -80°C .

Fibrosis assays. We developed a multiplex, bead-based assay (FibroPlex version 2) to measure 36 different predictors, mediators, and outcomes of fibrosis. This assay builds upon our original assay (FibroPlex), which contained TGF- β 1, TIMP-1, macrophage inflammatory protein 1 α (MIP-1 α), IL-13R α 2, bone morphogenic protein 7 (BMP-7), CTGF, matrix metalloproteinase 1 (MMP-1), and IL-13 (9). We expanded this assay to include additional key predictors or regulators of hepatic and pulmonary fibrosis. Additional analytes include insulin-like growth factor 1 (IGF-1), collagen IV, collagen VI, procollagen I carboxy-terminal propeptide (PICP), amino-telopeptide of type I collagen (ICTP), desmosine, pyridinoline (PYD), IGF binding protein 3 (IGFBP-3), IGFBP-5, thrombospondin (TSP1), MMP-2, MMP-7, MMP-8, MMP-9, endothelin-1, α -defensin, TIMP-2, TIMP-3, TIMP-4, tenascin C (TN-C), syndecan 1, Fas ligand, osteopontin, surfactant pulmonary-associated protein (SP-D), chemokine ligand 18 (CCL-18), MCP-1, and laminin (see Table S1 in the supplemental material). To maintain interanalyte cross-reactivity below 10% (9), these analytes are divided into 5 individual multiplexed assays that are performed simultaneously on a high-speed pipetting robot (Tecan Systems, Inc., San Jose, CA). Cord serum samples were analyzed using a BioPlex bead system (Bio-Rad, Hercules, CA). The low limit of detection for each analyte is shown in Table S1.

Statistical analysis. All analyses were performed using JMP Pro version 10 (SAS Institute, Cary, NC). Statistical significance was defined as *P* values of <0.05. Fibrotic markers and birth weight were evaluated as

continuous variables. Maternal schistosome infection status, SGA, and prematurity were evaluated as nominal variables (yes/no). The relationship between maternal schistosome infection status and each fibrotic marker was examined using multivariate linear regression. Potential confounders included coinfection with *A. lumbricoides*, *T. trichiura*, or hookworm, body mass index, maternal age, gravida, parity, a history of miscarriage, a history of stillbirth, smoking status, SES, birth weight, and gestational age.

We performed bivariate analysis of maternal schistosome infection status for each potential confounder. Potential confounders that were significantly related to maternal infection status were evaluated for inclusion in multivariate models exploring the relationship between FibroPlex version 2 markers and infection status. Potential confounders significantly related to any of the birth outcomes were evaluated for inclusion in the individual regression models created for each birth outcome (low birth weight [LBW], prematurity, SGA, or birth weight).

RESULTS

Demographic, parasitological, and gestational characteristics of the included subjects are shown in Table 1. Of the 150 enrolled pregnant women, we obtained a sufficient volume of cord serum from 109 subjects for assessment of fibrotic markers of interest. These subjects (*n* = 109) did not differ in any baseline demographic, parasitological, or gestational characteristics from the 41 women without sufficient cord serum (data not shown). Therefore, all subsequent analyses presented were performed on these 109 subjects. Of these, 56 of the mothers were found to be positive for *S. japonicum* infection (51%), while 53 were free of schistosome infection (49%). The infected women differed on numerous parameters from their uninfected counterparts. These include coinfection with *A. lumbricoides* (45% compared to 31%), hookworm (29% compared to 11%), age (29.5 compared to 32.7 years), and SES (14.4 compared to 16.2) in infected compared to uninfected women, respectively. These potential confounders guided the development of multivariate linear regression models assessing the relationship between *S. japonicum* infection status and specific fibrotic markers. In addition, the directionality of the effect of SES on levels of specific fibrotic markers changed depending on schistosome infection status, indicating an interaction between SES and infection status on the levels of some fibrotic markers. Hence, the term schistosome infection (*y/n*)*SES was included in the linear regression model.

We measured a total of 35 different proteins in cord serum. After we adjusted for potential confounders and interaction between SES and infection status, 10 distinct proteins were significantly elevated in the cord blood of neonates born to schistosome-infected compared to uninfected mothers. The remaining 25 molecules (collagen IV, PYD, IGFBP-3, IGFBP-5, TSP, ET-1, α -defensin, IL-13, IL-13R α 2, TIMP-2, TIMP-3, MMP-1, MMP-7, MMP-8, MMP-9, TN-C, syndecan 1, Fas ligand, osteopontin, SP-D, BMP-7, MIP-1 α , CCL18, MCP-1, laminin) were not significantly different in the cord blood of infants born to infected mothers compared to those of uninfected controls.

Markers of ECM metabolism are increased in the cord blood of infants from schistosome-infected mothers. We measured PICP as a marker of collagen synthesis and ICTP as a marker of collagen degradation (22), in addition to a number of other regulators of ECM remodeling (MMPs and TIMPs). Soluble levels of specific components of the ECM were also measured in the cord serum of these infants. After adjustment for confounders, PICP, ICTP, MMP-2, TIMP-1, and TIMP-4 levels

TABLE 1 Characteristics of the study population, stratified by schistosome infection status

Characteristic ^a	Value		P value
	<i>S. japonicum</i> infected (n = 56)	<i>S. japonicum</i> uninfected (n = 53)	
Median <i>S. japonicum</i> egg count in epg (IQR)	24.2 (10, 85.8)	0 (0)	<0.001
% (no.) of <i>S. japonicum</i> low-intensity infection (1–99 epg)	79 (44)	0 (0)	<0.001
% (no.) of <i>S. japonicum</i> moderate-intensity infection (100–399 epg)	21 (12)	0 (0)	<0.001
% (no.) <i>Ascaris lumbricoides</i> infected	80 (45)	58 (31)	0.02
% (no.) <i>Trichuris trichiura</i> infected	80 (45)	74 (39)	0.50
% (no.) hookworm infected	52 (29)	21 (11)	0.001
Median body mass index (IQR)	21.5 (19.4, 24.4)	22.2 (20.1, 24.1)	0.42
Mean maternal age in yrs (95% CI)	29.5 (27.7, 31.2)	32.7 (30.8, 34.6)	0.01
Median gravida (IQR)	3 (2, 5)	4 (2, 6)	0.21
Median parity (IQR)	2 (1, 4)	2 (1, 5)	0.24
% (no.) with history of miscarriage	10.7 (6)	9.6 (5)	1.0
% (no.) with history of stillbirth	3.6 (2)	3.8 (2)	1.0
% (no.) of smokers	0 (0)	3.8 (2)	0.23
Mean socioeconomic status score (95% CI)	14.4 (13.1, 15.6)	16.2 (15.0, 17.5)	0.04
Mean birth weight in kg (95% CI)	2.9 (2.8, 3.0)	2.9 (2.8, 3.0)	0.70
Median gestational age in wks (IQR)	40.0 (38.0, 40.3)	39.0 (38.0, 40.0)	0.15
% (no.) with low birth weight	7.1 (4)	15.7 (8)	0.22
% (no.) with intrauterine growth restriction	17.9 (10)	27.5 (14)	0.26

^a IQR, interquartile range; CI, confidence interval.

were all significantly higher in the cord serum from infected pregnancies than in uninfected pregnancies (1.6-, 1.5-, 4.4-, 1.6-, and 1.5-fold higher, respectively, all $P < 0.01$; Fig. 1A to E). In addition, the levels of collagen VI and desmosine in cord serum were also higher in neonates from infected mothers than

in neonates from uninfected mothers (1.3- and 1.8-fold higher; Fig. 1F and G). Of these, only MMP-2 was significantly positively correlated with intensity of infection (data not shown). Interestingly, while the PICP/ICTP ratio (collagen deposition/collagen catabolism) is not different between the two groups, the MMP-2/TIMP-4 ratio (collagen catabolism/inhibitor of collagen catabolism) is higher among neonates born to mothers with schistosomiasis during pregnancy than among those born to uninfected mothers (Fig. 1H).

Growth factors associated with the promotion of fibrosis are increased in the cord blood of infants from mothers with schistosomiasis. FibroPlex version 2 measures several growth factors associated with fibrosis. IGF-1, TGF- β 1, and CTGF were significantly higher in the cord serum from neonates exposed to schistosomiasis *in utero* than from neonates not exposed, after adjusting for the confounders included in our linear regression model (1.4-, 2.0-, and 2.7-fold higher, respectively; Fig. 2). In addition, CTGF was significantly positively correlated with intensity of infection (data not shown). All three of these growth factors have been implicated in the development of fibrosis in other physiological systems (23, 24).

Prematurity and birth weight are associated with specific markers of ECM metabolism and fibrosis-associated growth factors. We evaluated the relationship between each of the fibrosis-associated molecules included in the FibroPlex version 2 and specific birth outcomes, including a diagnosis of low birth weight (LBW), SGA, prematurity, and birth weight. After adjusting for smoking status using linear regression, a number of the same proteins that were elevated in the cord blood from schistosome-infected pregnancies were also elevated in pregnancies with premature delivery of the neonate. These include PICP, ICTP, desmosine, MMP-2, TIMP-1, TGF- β 1, and CTGF (Fig. 3).

In addition, after controlling for smoking status and parity, infants with cord blood CTGF levels in the upper tertile of the

FIG 1 Soluble mediators of ECM remodeling are elevated in the cord blood of neonates born to *S. japonicum*-infected mothers. Soluble mediators of ECM remodeling were measured in cord blood samples taken at delivery using a multiplexed bead-based assay. Procollagen I carboxy-terminal propeptide (A; $P = 0.014$), amino-terminal propeptide of type I collagen (B; $P = 0.007$), MMP-2 (C; $P = 0.001$), TIMP-1 (D; $P = 0.001$), TIMP-4 (E; $P = 0.005$), collagen VI (F; $P = 0.005$), and desmosine (G; $P = 0.006$) are all elevated in infants born to *S. japonicum*-infected mothers. The ratio of MMP-2 to TIMP-4 was also elevated in these children (H; $P = 0.03$). Data are represented as means $\pm$ standard errors of the means (SEM).

FIG 2 Profibrotic growth factors are elevated in the cord blood of neonates born to *S. japonicum*-infected mothers. Profibrotic growth factors were measured in cord blood samples taken at delivery using a multiplexed bead-based assay. IGF-1 (A; $P = 0.016$), TGF-β1 (B; $P = 0.012$), and CTGF (C; $P = 0.041$) are all elevated in samples from neonates with infected mothers compared to their uninfected counterparts. Data are represented as means $\pm$ SEM.

distribution were born an average 209 g lighter than infants with CTGF levels in the low and middle tertiles ($P = 0.002$; Fig. 4).

DISCUSSION

Schistosomiasis remains a critical public health problem in areas where it is endemic, with schistosome-associated fibrosis com-

FIG 4 CTGF in cord blood is associated with decreased birth weight. After controlling for smoking status and parity, infants with the detectable levels of cord blood CTGF were born 168 g lighter than infants with undetectable levels of cord blood CTGF. Data are represented as means $\pm$ SEM.

prising its most morbid sequela. Because cycles of infection, treatment, and reinfection characterize the infection pattern in many areas where schistosomiasis is endemic, tools to identify individuals at risk of clinically significant fibrosis are urgently needed. In addition, praziquantel treatment is withheld from pregnant women in many areas where schistosomiasis is endemic; thus, identification of the impact of maternal schistosomiasis on pregnancy outcomes has heightened importance.

To identify biomarkers of current and future risk of fibrosis, we developed a multiplexed bead-based assay (FibroPlex version 2) which simultaneously quantifies 35 markers of fibrosis. All of these markers were evaluated in cord blood from pregnancies with and without maternal schistosome infection. Each of the 10 schistosome-associated profibrotic proteins identified in this report has been associated with fibrosis in children and nonpregnant

FIG 3 Profibrotic markers in cord blood are associated with premature birth. Multiple profibrotic markers are also elevated in the cord blood from pregnancies that experienced preterm birth. Associations were assessed with linear regression after correcting for smoking status. Data are represented as means $\pm$ SEM and include PICP (A; $P = 0.003$), ICTP (B; $P = 0.02$), MMP-2 (C; $P < 0.01$), TGF-β1 (D; $P < 0.0001$), desmosine (E; $P = 0.001$), CTGF (F; $P < 0.01$), and TIMP-1 (G; $P = 0.02$).

adults with schistosomiasis (9, 25–27). However, the elevation of these markers in neonates is remarkable, because there is no evidence for vertical transmission of schistosomiasis japonica in humans, despite its occurrence in other mammals with very different placental morphology (28). There are several possible mechanisms to explain the upregulation of these profibrotic markers in cord blood, including transplacental transport of maternally derived fibrotic markers. This possibility, however, is unlikely, as active transplacental transport of markers of collagen metabolism has not been reported in humans. Alternatively, the upregulation of these profibrotic markers in cord blood may reflect a fetal response to transplacentally transported schistosome antigens.

There is extensive evidence in humans documenting both the transplacental transport of schistosome antigens (11, 13–16) and consequent schistosome-specific adaptive immune responses in the cord blood of neonates of schistosome-infected mothers (12, 16, 17, 29). Increased levels of schistosome antigen-specific IgE and B cells are found at birth in these children, compared to those of unexposed controls (12, 29). Because of this transport, the initial exposure of many neonates to schistosome antigens in regions where schistosomiasis is endemic occurs *in utero*, potentially years before they acquire active infection. The impact of this sensitization on fibrosis-related outcomes as these infants acquire natural infections remains unknown. Multiple studies have shown that hepatomegaly and fibrosis associated with schistosome infection continue to affect children and adults even after initial infection is resolved (30–32). Because fibrosis is a morbidity associated with chronic schistosome infection, it is relatively rare in children (30, 33, 34); however, we speculate that the risk of developing schistosome-associated fibrosis following acquisition of infection may differ in individuals who were sensitized *in utero* to respond to schistosome antigens with a profibrotic signature.

In addition to their potential impact on natural infections acquired after birth and subsequent disease progression, many of the fibrosis-associated molecules have been linked with adverse birth outcomes, including intrauterine growth restriction (IUGR), preterm birth, and preeclampsia. The roles of PICP and ICTP, as markers of bone matrix turnover, have been studied in the neonate primarily in the context of fetal growth regulation as it relates to infants born with IUGR or SGA. However, they are also elevated in infants born prematurely, compared to those of full-term infants (35, 36), suggesting that global collagen turnover is increased in preterm infants. In addition, MMP-2 and -3 are elevated in preterm infants, which may reflect increased ECM degradation associated with preterm labor (37). Specific growth factors and cytokines are also elevated in infants born prematurely, including many proinflammatory cytokines and the growth factor TGF- β 1 (38). Thus, our data relating schistosome-associated increases in profibrotic markers and preterm birth are consistent with the literature focusing on mediators of preterm birth.

Importantly, there is no evidence for transport of profibrotic proteins across the placenta, supporting the notion that the changes observed in the cord blood of neonates from schistosome-infected mothers are of fetal origin and not due to maternal transport (39). Elevated levels of these molecules suggest that ECM remodeling is more dynamic in infants born to infected mothers. Concordant with these data, the ratio of MMP-2 to TIMP-4, a key collagen catabolic protease/inhibitor system, was markedly elevated in the cord blood from infants born to schisto-

some-infected mothers compared to that of uninfected mothers, again suggesting a higher degree of collagen turnover in these infants.

CTGF levels in cord blood have been related to fetal growth restriction in the context of preeclampsia (40), although its precise role at the maternal-fetal interface in this scenario remains unknown. These reports echo our findings that infants with the detectable levels of cord blood CTGF were born 168 g lighter than infants with no detectable levels of cord blood CTGF and may also reflect the association between CTGF and prematurity observed in our study population.

To our knowledge, this is the first report identifying a profibrotic signature in neonates born to schistosome-infected mothers. We are currently planning long-term follow-up studies of these children as well as children participating in an ongoing randomized control trial of praziquantel treatment during pregnancy (ClinicalTrials.gov, registered study number NCT00486863) to assess the impact of *in utero* exposure to schistosome antigens on fibrosis in offspring who acquire active schistosome infections early in life.

ACKNOWLEDGMENTS

We thank Peter Marinkovich and Arthur Veis for their generous sharing of antibodies for inclusion in this assay.

This work was supported by the National Institute of Allergy and Infectious Diseases (1F32AI093043 to E.A.M., R01AI48123 to J.D.K., U01AI066050 to J.F.F., 1R01AI076353 to E.S.W.) and the National Heart, Lung and Blood Institute (HL109118-01A1 to E.S.W.) at the National Institutes of Health.

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