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Schistosoma mansoni: Egg-induced downregulation of hepatic stellate cell activation and fibrogenesis

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ABSTRACT

Eggs of *Schistosoma mansoni* trapped in human liver can lead to fibrosis. Since liver fibrosis requires activation of hepatic stellate cells (HSC) from a quiescent to a myofibroblastic phenotype, we investigated the effects of *S. mansoni* eggs on this process using *in vitro* co-cultures with human HSC and evaluated established biomarkers for activation and fibrosis. HSC demonstrate significantly reduced expression of α-smooth muscle actin ($p < 0.001$), connective tissue growth factor ($p < 0.01$) and type I collagen ($p < 0.001$) but significantly increased expression of peroxisome proliferator-activated receptor-γ ($p < 0.01$). Morphologically, HSC exhibited elongated fine cellular processes and reduced size, increased accumulation of lipid droplets and reduced expression and organization of α-smooth muscle actin and F-actin stress fibres. Additionally, schistosome eggs prevented the HSC fibrogenic response to exogenous transforming growth factor-β. In summary, schistosome eggs blocked fibrogenesis in HSC, a finding which may have implications for our understanding of the fibrotic pathology in *S. mansoni* infections.

1. Introduction

Schistosomiasis is an important helminth infection of man. It is estimated to infect 200 million people in the world (Crompton, 1999; Chitsulo et al., 2000), with 120 million symptomatic (Chitsulo et al., 2000), 20 million people with severe morbidity (Crompton, 1999) and 20,000 deaths a year (Crompton, 1999) resulting in loss of 4.5 million disability adjusted life years (DALYs) (WHO, 2002). The two most important species with regards to human liver disease are *Schistosoma mansoni* and *Schistosoma japonicum*. *S mansoni* is estimated to infect 83 million people in Africa, Caribbean and east Mediterranean regions (Crompton, 1999). Adult pairs of *S. mansoni* worms reside within the mesenteric veins where females release on average 340 eggs per female per day with rates ranging between 190 and 658 depending upon strain and experimental host used (Loker, 1983). More than 50% of eggs are carried to the liver by the portal circulation where they become trapped in the liver sinusoids (Wynn et al., 2004). The host immune response to the eggs in the liver is well documented and typically involves granuloma formation at the egg sites which can result in fibrosis and portal hypertension (Booth et al., 2004; de Jesus et al., 2004; Wynn et al., 2004).

In the liver, the cells primarily responsible for fibrogenesis are the hepatic stellate cells (HSC). The HSC is located in the space of Disse in the liver sinusoid and is responsible for maintenance of the extracellular matrix (ECM) (Sato et al., 1998), storage of vitamin A (Blomhoff and Wake, 1991), and has also been demonstrated to have a possible role in controlling blood flow through the liver (Reynaert et al., 2002). In response to liver injury, normally quiescent HSC are activated and undergo a process of transdifferentiation towards a myofibroblastic phenotype (Friedman, 2000). Adoption of this phenotype is associated with transforming growth factor (TGF)-β (Dooley et al., 2001a,b; Leask and Abraham, 2004), most notably with isoform 1 (Wickert et al., 2002). The fibrotic response of HSC to TGF-β1 stimulation is well documented and has been widely used as a model for *in vitro* HSC transdifferentiation studies (Dooley et al., 2001a,b). Myofibroblasts are characterized by their increased ECM production, contractility, expression of αSMA and loss of vitamin A storage (Friedman, 2000, 2003; Dooley et al., 2001a,b). The wound healing response is terminated when myofibroblasts are removed from the area at the end of the insult either by apoptosis or reversion back to a quiescent phenotype, however, during fibrogenesis this process fails (Friedman, 2003). A number of studies report that agonists of the peroxisome proliferator-activated receptor (PPAR)γ can block and reverse the process of transdifferentiation in HSC (She et al., 2005; Tsukamoto, 2005; Zhao et al., 2006), by a mechanism that appears to prevent TGF-β1-mediated signalling (Zhao et al., 2006). Key features of

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the PPARγ-mediated quiescence of the cells are lipid retention and a reduction of collagen production (She et al., 2005; Zhao et al., 2006). In addition, treatment with adipogenic factors has also been shown to restore the quiescent phenotype in primary rat cells (She et al., 2005) and the human LX-2 cell line (Zhao et al., 2006). The LX-2 cell line is a recently developed immortalised human HSC cell line (Xu et al., 2005) that retains the key features of primary HSC. As these cells are readily activated by culture on plastic they can be manipulated between quiescent and activated states and therefore provide a useful model for investigating HSC transdifferentiation. Human HSC have not been used widely in research as it is difficult to isolate them from tissue due to the infrequent availability of tissue that is suitable for cell isolation (Xu et al., 2005).

To date only a few studies have investigated the interactions between schistosome eggs and host cells. *S. mansoni* eggs or their crude soluble extracts have been found to stimulate hepatic endothelial cell proliferation (Freedman and Ottesen, 1988), migration and angiogenesis (Kanse et al., 2005) and to induce fibroblast proliferation (Wyler and Tracy, 1982) and collagen synthesis (Boros and Lande, 1983). Even less well understood is the role of HSC in schistosomiasis. It is thought that the HSC is one of the main sources of collagen deposition and ECM remodelling in schistosomiasis (Booth et al., 2004) and most recently, HSC have been identified in the periphery of egg granulomas in human *S. japonicum* infections and myofibroblasts were observed in end-stage human disease associated with fibrosis (Bartley et al., 2006). However, despite these findings and the *in vivo* HSC and egg co-localisation, there are no prior studies in which the direct effects of parasite eggs on these cells are considered.

This study was designed to investigate the nature of *S. mansoni* egg activity on human HSC using an LX-2 cell model. Specifically, morphological and transcriptional biomarkers of HSC activation and fibrogenesis were examined with HSC cultured on plastic in the presence of adipogenic factors or transforming growth factor (TGF)-β.

2. Materials and methods

2.1. Egg isolation

The parasite material was kindly provided by Prof. Alan Wilson, University of York, UK. Male C57BL/6xCBA mice were each infected with 180 *S. mansoni* cercariae via the shaved abdomen (Smithers and Terry, 1965). Seven weeks later the livers were removed, homogenised and digested with trypsin (Sigma–Aldrich, Poole, UK) for 3 h at 37 °C. The homogenate, containing the eggs was passed through a series of sieves (300–180 μm), the eggs collected by sedimentation then cleaned by washing six times in sterile phosphate-buffered saline. The clean eggs were then counted and resuspended in DMEM (Lonza, Wokingham, UK) cell culture medium. Eggs were stored in DMEM with 10% fetal bovine serum (FBS, Lonza) overnight before use the next day. Non-viable intact eggs were obtained by freezing them in PBS at −80 °C. Viability was assessed by monitoring the ability of eggs to hatch in fresh water. HSC were then co-cultured for up to 3 days in the presence of viable eggs at concentrations of 500, 1000 or 1500 eggs/ml. At these concentrations previous studies have elicited host cell responses from whole eggs, or from the equivalent concentrations of extracted soluble egg antigens (SEA). Accordingly, approximately 10 μg/ml SEA corresponds with 1000 eggs.

2.2. Cell cultures

LX-2 cells were a kind gift of Prof. Friedman, Mount Sinai School of Medicine, New York, USA. Cultures were maintained in DMEM containing 2% FBS plus antibiotics (complete medium) at 37 °C and with 5% CO_2. Medium was changed after 48 h. Cells were seeded in 24 well plates (Greiner bio-one, Stonehouse, UK) at a density of 4.3×10^3 cells per cm^2. For collagen gel experiments a reconstitution buffer was made with 0.2 ml 1N NaOH, 0.5 ml 7.5% $NaHCO_3$ and 0.3 ml of dH_2O. One hundred and twenty milliliters of this buffer was mixed with 120 μl of 10× DMEM and 960 μl of type 1 rat tail collagen to make the gel mixture. In a 35 mm plate 1 ml of gel was used and in a 24 well plate 300 μl was used. The gel was allowed to set at 37 °C before use. For co-culture experiments with parasite eggs, cells were treated for up to 3 days with either complete medium alone or complete medium + *S. mansoni* eggs (at concentrations of 500, 1000 and 1500 eggs/ml). For adipogenic differentiation cells were either treated with complete medium alone or complete medium + adipogenic factors, comprised of 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 1 μM insulin (Sigma–Aldrich) and compared to complete medium + *S. mansoni* eggs (1000 eggs/ml). For experiments with recombinant TGF-β1 (Peprotech EC, London, UK), cells were treated with either complete medium + TGF-β1 (at a concentration of 5 ng/ml) or with complete medium + *S. mansoni* eggs (1000 eggs/ml), + TGF-β1 (5 ng/ml).

2.3. Immunofluorescence and phase-contrast microscopy

Cells were fixed in methanol for immunofluorescence then washed three times in PBS. A mouse monoclonal anti-human αSMA antibody (Sigma–Aldrich, clone 1A4) and Texas-Red conjugated anti-mouse IgG antibody (Vector Laboratories, Peterborough, UK) were employed for detection of αSMA expression. For this the fixed cells were washed with PBS before a blocking solution of 5% bovine serum albumin (BSA, Sigma–Aldrich) was added to the cells. This was removed and the cells incubated at room temperature with the primary antibody (1:800 in 5% BSA/PBS) for 1 h. This was then removed and the cells washed in PBS before the cells were then incubated at room temperature in the dark with the secondary antibody (1:300 in 5% BSA/PBS) for 45 min. Cells were then washed in PBS and nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI, Vector Laboratories). For staining of F-actin, cells were fixed in 3.7% formaldehyde solution in PBS after a prewash with PBS. The cells were then washed with PBS and left at −20 °C in acetone to permeabilise. The cells were again washed in PBS before a staining solution containing FITC-conjugated phalloidin (Invitrogen, Paisley, UK) diluted 1:50 in 1% BSA was applied for 20 min. Cells were counterstained with DAPI as before. Phase-contrast imaging was used for general cell morphology and for detection of cellular lipids. Cells for lipid droplet staining were fixed for 30 min in 2% paraformaldehyde (Sigma–Aldrich) and washed in dH_2O before incubation with 99% (v/v) propane 1,2-diol (Sigma–Aldrich) for 5 min. This was removed and Oil-Red O (0.5% w/v) in propane 1,2-diol was added to the cells for 30 min. Oil-Red O solution was removed and the cells washed for 5 min with 85% (v/v) propane 1,2-diol. This was removed and the cells washed with dH_2O. Cells were then counterstained with Haematoxylin (Vector Laboratories). Phase-contrast and immunofluorescence images were obtained with a Nikon TE2000 Eclipse inverted fluorescence microscope with a cooled Hamamatsu Orca camera system and merged using Image-Pro Lab v3.7 image analysis software (Nikon UK Ltd., Kingston upon Thames, UK). Images of Oil-Red O stained lipid droplets were obtained using a Nikon Coolpix 4500 camera attached to the Nikon TE2000 microscope.

2.4. Realtime PCR

Primers and probes targeting expression of type I collagen (Col1A1), connective tissue growth factor (CTGF), and peroxisome