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ORIGINAL ARTICLE

Tumourigenic effect of *Schistosoma haematobium* total antigen in mammalian cells

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Summary

Schistosoma haematobium is endemic in several regions of Africa and has been shown to be associated with predominantly squamous cell bladder carcinoma. The mechanisms underlying the association between *S. haematobium* and bladder squamous cell carcinoma is largely unknown. All the reports so far, demonstrate exclusively an epidemiological evidence linking *S. haematobium* infection with squamous cell bladder carcinoma. We hypothesized that these parasite antigens might induce tumourigenesis. For this, we used normal mammalian cells of Chinese hamster ovary (CHO) and treated the cells in culture with *S. haematobium* total antigen (Sh). Our results showed increased proliferation in Sh-treated cells in comparison with the controls. The CHO cells exposed to Sh were inoculated subcutaneously into male nude mice and formed sarcomas ($n = 5/5$). The cells from the sarcomas expressed vimentin filaments and were negative to cytokeratin. Our results demonstrate for the first time that *S. haematobium* antigens induce tumour development in nude mice.

Keywords
Schistosoma haematobium, Chinese hamster ovary cell line, cell proliferation, nude mice, argyrophilic nucleolar organizing region

Human urothelial carcinomas constitute the vast majority of bladder cancers in most of the world. Smoking, exposure to certain dyestuffs, aromatic amines, rubber production, painting and leatherwork seem to be associated with an increase risk of bladder cancer in developed countries (Kogevinas & Trichopoulos 2002). *Schistosoma haematobium* is endemic in several regions of Africa and has been shown to be associated with predominantly squamous cell

bladder carcinoma, a rare subtype in the western world (Gutiérrez *et al.* 2004). The epidemiological association between *S. haematobium* and squamous cell carcinoma is based both on case control studies and on the close correlation of bladder cancer incidence with prevalence of *S. haematobium* infection within different geographical areas. The mechanisms in which urinary schistosomiasis induces bladder cancer are incompletely understood. Both chemical and physical carcinogenic pathways have been implicated and it has been suggested that chronic mechanical irritation by calcified eggs deposited in the bladder epithelium invokes changes in the urinary tract epithelium (Hodder *et al.* 2000). Chinese hamster ovary cells (CHO cells) are a cell line derived from Chinese hamster ovary cells often used in biological and medical research. We hypothesized that the parasite total antigen might induce tumourigenesis. In this study, we employed a cell biology approach to investigate the involvement of *S. haematobium* total antigen in tumourigenesis. In this paper, we document evidence for the oncogenic potential of *S. haematobium* total antigen by its treatment of CHO cells resulting in increased proliferation and tumourigenesis in nude mice. To characterize the tumours developed in mice, we made a histological study and examined argyrophilic nucleolar organizing region (AgNOR), cytokeratin and vimentin expression. To our knowledge, this is the first report demonstrating alterations of normal mammalian cells and tumourigenesis as a direct effect of *S. haematobium* antigens. Strategies to block these antigens may contribute to halt the growth of squamous cell bladder carcinomas associated with *S. haematobium*.

Methods

Schistosoma haematobium total antigen production

Schistosoma haematobium adult worms were collected by perfusion of the hepatic portal system of golden hamsters at 7 weeks after infection with 100 cercariae. The worms were suspended in phosphate-buffered saline (PBS) and then sonicated. The protein extract was then ultracentrifuged and the protein concentration was estimated using a micro BCA (bicinchoninic acid) protein assay reagent kit.

Cell culture

Chinese hamster ovary (CHO) cell line was obtained from IPATIMUP. Chinese hamster ovary cells were cultured and maintained at 37 °C in a 5% CO2 humidified atmosphere in CHO medium (Sigma) with 10% FBS (Fetal Bovine Serum) and 1% penicillin/streptomycin (Sigma). Cells were passaged every 5 days. Before treatments, cells were serum-starved for 16 h. The cells were treated with Sh for 24 h. Control cells were treated with serum free medium. After treatment, the cells were harvested by trypsinization and counted under microscope after trypan blue staining.

Proliferation assay

The CellTiter 96 AQ non-radioactive cell-proliferation assay (Promega, Madison, WI, USA) was used to assess cell proliferation. The assay is composed of the tetrazolium compound MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent, PES (phenazine ethosulfate), to produce a colorimetric change. MTS is reduced by viable cells to formazan, which can be measured with a spectrophotometer by the amount of 490 nm absorbance. Formazan production is time dependent and proportional to the number of viable cells. Chinese hamster ovary cells were cultured in 0.1 ml CHO media in 96-well flat-bottomed plates. Cultures were seeded at 1×10^4 cells/well and allowed to attach overnight. After the indicated time of incubation with the appropriate medium, 20 μl reagent was added per well, and cells were incubated 1 h before measuring absorbance at 490 nm. Background absorbance from the control wells was subtracted. Studies were performed in triplicate for each experimental condition.

Nude mice

The experiments were carried out in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for experimental and other scientific purposes and related European Legislation (OJ L 222, 24.8.1999). Male N:NIH(s)II-nu/nu mice of 4–6 weeks old were obtained from IPAT-IMUP. These animals were maintained under sterile conditions throughout the experiment (temperature 24 ± 2 °C, relative humidity 55 ± 5% and a 12 h photoperiod) in polycarbonate cages. They were fed sterilized autoclave rodent feed and water *ad libitum*.

Tumourigenesis

Eight mice were randomly divided into two experimental groups, group 1 with five animals and group 2 with three animals. Chinese hamster ovary cells were suspended in 200 μl of PBS and inoculated subcutaneous in the neck into the mice from group 1. Animals from group 2 were used as

the control group; these animals were injected with CHO cells not treated with Sh.

After transplantation, the size of each tumour mass developed was measured with a calliper twice a week and tumour volumes were calculated as previously described (Tang *et al.* 2003). On day 22 after cell administration, all animals were killed under anaesthesia by intracardiac injection of pentobarbital (40 mg/kg) and tumours were removed from each animal. The tumours were examined macroscopically, cut in sections with 4 mm, fixed in 10% formalin and then embedded in paraffin. Haematoxylin and eosin (H&E) stained sections were prepared and examined to classify the neoplasic transformation.

Immunohistochemical procedure and evaluation

Immunohistochemistry was carried out with the streptavidin–biotin-peroxidase complex technique as previously described (Oliveira *et al.* 2008) using Ultravision detection system anti-polyvalent, HRP (LabVision Corporation, Fremont, CA, USA) for pan Cytokeratin, Cytokeratin (CK) 7, CK 20, Anti-Cytokeratin (CAM 5.2) and vimentin, using specific primary antibodies raised against pan-cytokeratin (cocktail of cytokeratins 5, 6, 8 and 18, clones 5D3 and LP53; Abcam Inc, Cambridge, MA, USA) diluted 1:100, CK 7 (clone OV-TL 12/30; Neomarkers, Fremont, California, USA) diluted 1:100, CK 20 (clone Ks20.8; Neomarkers) diluted 1:20, CAM 5.2 (clone CAM 5.2; Becton Dickinson, Franklin Lakes, NJ, USA) diluted 1:5 and vimentin (clone V9; Neomarkers) diluted 1:100. Briefly, deparaffinized and rehydrated sections were immersed in PBS. Slides were then incubated with 3% hydrogen peroxide in methanol for 10 min. Slides were incubated in Ultravision block solution (Neomarkers) for 10 min at room temperature before overnight incubation with the primary antibody. Then, sections were sequentially washed in 1× PBS with 0.02% Tween-20 incubated with biotinylated goat anti-polyvalent antibody for 10 min, streptavidin peroxidase for 10 min, and developed with 3,3-diaminobenzidine for 10 min. Slides were counterstained with Mayer's haematoxylin (Merck, Dermstadt, Germany) and mounted with entelan (Merck). Negative controls were carried out by omitting the primary antibody.

Cytokeratins and vimentin expression were evaluated as positive or negative, independent of marker extension.

Argyrophilic nucleolar organizing region histochemical procedure and evaluation

Argyrophilic nucleolar organizing region (AgNOR) histochemical staining was performed using a previously described modified 1-step silver-staining technique (Ploton *et al.* 1986). In brief, 2-μm sections of formalin-fixed paraffin-embedded tissue were cut, deparaffinized in xylene, rehydrated in graded ethanol and rinsed in distilled water. Slides were incubated for 30 min at room temperature in the dark with freshly made AgNOR staining solution consisting of 0.02-g gelatin in 1 ml of 1% formic acid and 1-g silver nitrate in 2 ml of distilled water. Following AgNOR staining, slides were rinsed with distilled water, dehydrated with graded ethanol and xylene and coverslipped.

To determine the average AgNOR count/cell in each tumour, AgNORs were counted in 1000 randomly selected neoplastic cells throughout the tumour at 1000× magnification. Individual AgNORs were resolved by focusing up and down while counting within individual nuclei. Average AgNOR counts/cells were then determined on the basis of averaging the counts within these 1000 random neoplastic cells as has been shown by numerous publications (Martin *et al.* 1992).

Statistical analysis

All statistical tests were performed using the SPSS 12 statistical software (SPSS Inc. USA). The results are presented as mean ± standard deviation. The values of AgNOR for various groups were compared using Student's *t*-test.

Results

Schistosoma haematobium *total antigen increased the proliferation of CHO cells* in vitro

To begin investigating the effect of *Schistosoma haematobium* total antigen (Sh) on cell viability and proliferation, CHO cells were seeded on 96 well plates, starved overnight, treated with increasing concentrations of Sh for 24 h, cultivated for 24, 48 and 72 h and then analysed by MTS assay (Figure 1). The growth curve shows that treated cells proliferated significantly faster and more than the control cells. These results suggest that the increased proliferation in CHO cells is a consequence of Sh treatment. We used the concentration of 50 μg/ml of Sh for the subsequent assays.

Animal general condition

No deaths attributable to the treatment were observed. All mice from group 1 had positive tumour growth 15 days after inoculation and were necropsied 22 days later. There was no tumour growth in control group. Sh allows the vigorous growth of CHO xenografts in nude mice (Figure 2).

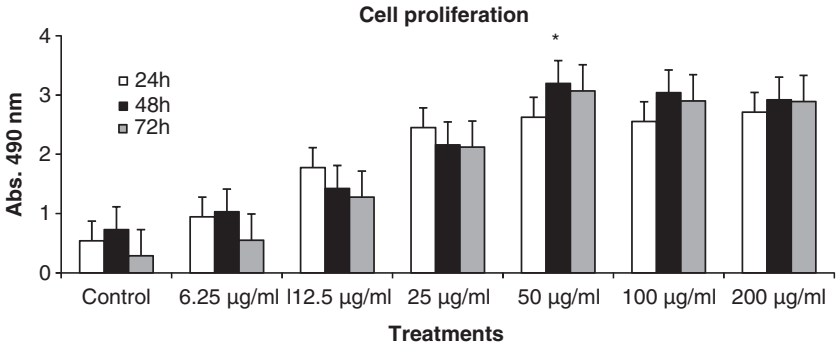

Figure 1 Cell proliferation assay of *Schistosoma haematobium* total antigen treated cells. The experiments were carried out in triplicate. The growth curve shows that treated cells proliferated significantly faster and more than the control cells. ($P < 0.01$; control *vs.* 50 µg/ml).

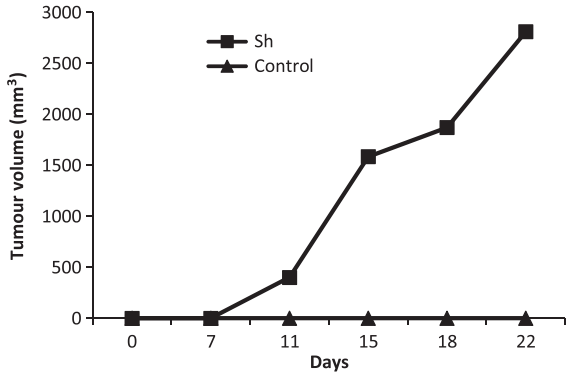

Figure 2 Analysis of the tumour volume induced by control Chinese hamster ovary (CHO) and *Schistosoma haematobium* total antigen (Sh) treated CHO in nude mice. Tumour volumes were measured at the indicated days after injection. Each point represents the mean of five individual determinations.

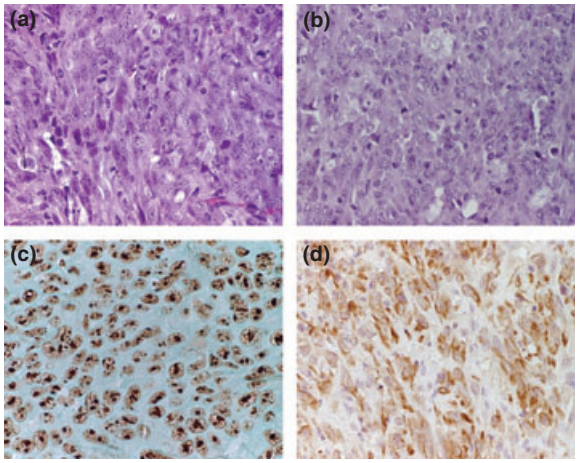

Figure 3 Histological findings of the tumour mass produced by inoculation of Chinese hamster ovary (CHO) cells exposed to *Schistosoma haematobium* antigen (a, b) mice sarcomas stained by Haematoxylin and eosin (H&E) (600×), (c) mice sarcoma stained by argyrophilic nucleolar organizing region (AgNOR) technique (600×) and (d) Immunohistochemistry of sarcoma showing vimentin filaments (600×).

Macroscopic and microscopic evaluation

All mice injected with Sh developed a solid, large multilobulated mass in interscapullary region. These tumours grew very fast and ulcerated overlying the skin. No macroscopic changes were observed in the interscapular region in the control group. The cut surface of the tumours was white and firm. Microscopic observation of these tumours showed a pleomorphic and fusocellular neoplasia with intensive inflammatory infiltrate, predominantly lymphocytes and neutrophils. We have also identified round cells with prominent nucleus, and exuberant nucleolus, with a histiocytic pattern and frequent mitosis. Necrotic foci were seen in these tumours. All tumours corresponded to sarcomas. There were no tumour metastases. Histological findings from mice inoculated with Sh can be observed in Figure 3.

Immunohistochemistry and histochemistry

Immunohistochemistry was performed on all tumours from group 1. These tumours strongly expressed vimentin filaments.

Table 1 Results of immunohistochemistry and histochemistry

		Immunohistochemistry		Histochemistry
Group	**Tumour**	**Cytokeratins**	**Vimentin**	**AgNORs**
1(Sh)	Sarcoma	–	+	1.97 ± 1.09*
1(Sh)	Sarcoma	–	+	1.94 ± 1.06
1(Sh)	Sarcoma	–	+	1.72 ± 0.9
1(Sh)	Sarcoma	–	+	1.88 ± 1.09
1(Sh)	Sarcoma	–	+	1.86 ± 1.12
2(control)	–	–	–	–
2(control)	–	–	–	–
2(control)	–	–	–	–

*Values are mean values ± SEM ($P > 0.05$, *t*-test); + positive, – negative.
Sh, *Schistosoma haematobium* total antigen; AgNORs, argyrophilic nucleolar organizing regions.

The tumours were negative for the tested cytokeratins (Table 1 and Figure 3). Argyrophilic nucleolar organizing regions were visible as black or brown dots, with various sizes, within the nuclei of cells (Figure 3). The mean values of AgNORs were similar and not statistically different between tumours, these values are shown in Table 1.

Discussion

Here, we have used a xenograft model to show that Sh has the potential to induce tumour development. First we used Sh in cell cultures and showed that Sh increases proliferation. We have no references so far in the literature to compare our results. However, the results of Wyler and Tracy (1982) clearly indicate that total antigen from *Schistosoma* eggs contains a biologically active molecule(s) capable of directly stimulating fibroblasts *in vitro*. It has also been shown that prolonged stimulation of excessive proliferation of urinary bladder epithelial cells in rats results in carcinoma formation (Otori *et al.* 1997). Schistosomal granulomas are a rich source of cytokines and growth factors, whose role in connective tissue proliferation and activation as well as in fibroplasias is well established. Extensive proliferation of inflammatory cells of myeloid origin associated with schistosomal granulomas is also well known (Chiarini *et al.* 2006).

Chinese hamster ovary cells exposed to Sh described in this paper formed similar phenotypes in all inoculated nude mice. It is worth noting that all the formed phenotypes were sarcomas. The morphology of CHO cells is both epithelial-like and fibroblast-like. Our results suggest that Sh may induce the transformation of the fibroblast-like component of CHO cells into sarcomas. This result is not surprising as it has been shown that egg granulomas isolated from livers of mice infected with *Schistosoma mansoni* or *S. japonicum* and cultured *in vitro* released a fibroblast-stimulating substance which stimulated proliferation in resting dermal fibroblasts (Wyler & Tracy 1982). Schistosomal egg granulomas spontaneously secrete fibrogenic factors, suggesting that there exists a molecular link between granulomatous inflammation and hepatic fibrosis in schistosomiasis (Prakash *et al.* 1990). Hepatic fibrosis complicates the chronic granulomatous inflammatory reaction to *S. mansoni* eggs and is the major cause of morbidity and mortality in human schistosomiasis (Wyler 1983). This liver pathology is the terminal event of a complex pathophysiological cascade involving interactions between fibroblasts and both host and parasite products (Lammie *et al.* 1986) and is associated with prominent accumulations of fibroblasts (Dunn *et al.* 1986), reinforcing the present results.

Carcinogenesis is a complex process in which normal cell growth is modified as a result of the interaction of multiple factors. There is a strong evidence to suggest that biological carcinogens are a major cause of cancer in humans. It has been estimated that chronic infections by virus, bacteria and parasites contribute to 13% of world's cancer (Parkin *et al.* 1999).

Although helminths have been implicated in the aetiology of human cancer, the knowledge of the mechanisms by which parasites induce malignant transformation of the host cells is unclear. *Schistosoma haematobium* is considered to be carcinogenic to humans, with sufficient evidence for its role in causing carcinoma of the bladder (Parkin 2006). According to Herrera and Ostrosky-Wegman (2001), parasites might initiate carcinogenesis by direct action of genotoxic factors, either secreted by them or produced during the inflammatory response.

Inflammation is a common feature of helminthiasis in which inflammatory cells regenerate reactive oxygen species that, apart from killing invading pathogens, are capable of inducing genetic instability in normal surrounding tissue, which can lead to malignant transformation. As we show with AgNOR evaluation, the sarcomas developed in nude mice had a high proliferation index. Several authors reported that AgNOR staining is a simple and useful method for estimating tumour cell proliferation (Khan *et al.* 2006). Nucleolar organizer regions are focal aggregates of intranucleolar non-histone proteins that are associated with potential sites of ribosomal DNA transcription (Rüschoff *et al.* 1989). These proteins are easily localized by virtue of their argyrophilia. The mean number of AgNORs per nucleus accurately correlates with mitotic rate in tumour cell lines and AgNOR counts may thus provide indirect measurement of mitotic rate in tumours.

Further investigations using *S. haematobium* antigens may be useful to understand tumourigenesis associated with this parasite.

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