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Fucoidan stimulates cell division in the amebocyte-producing organ of the schistosome-transmitting snail *Biomphalaria glabrata*

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Abstract

Adult Salvador (schistosome-resistant) strain *Biomphalaria glabrata* snails were injected with 5 μ l of 10 mg/ml solutions of the sulfated polysaccharides λ carageenan, dextran sulfate, fucoidan, and heparin, the nonsulfated polysaccharide laminarin, and the monosaccharides L-fucose and L-galactose, and mitotic activity in the amebocyte-producing organ (APO) was measured in histological sections at 24h post injection. Among the substances tested, only fucoidan induced elevated mitotic activity. Desulfated fucoidan was not mitogenic, indicating that sulfate groups are required for activity. Schistosome-susceptible M-line snails possessed minimal or no hematopoietic tissue in their APO, which did not respond to fucoidan. Immersion of juvenile Salvador snails in 1 or 10 mg/ml solutions of fucoidan for 3h did not elevate mitotic activity at 24h post immersion, suggesting that the external and digestive tract epithelia of *B. glabrata* are impermeable to this molecule. These results provide support for the hypothesis that fucosylated glycans on the tegument and in excretory-secretory products of sporocysts of *Schistosoma mansoni* are in part responsible for increased mitotic activity in the APO of *B. glabrata* infected with this trematode or injected with its extracts.

Keywords

fucosylated glycans; hematopoiesis; hemocytes; mitosis

Introduction

The majority of non-self receptors that have been described in molluscs are carbohydrate-binding proteins called lectins (Bayne, 2009). Among these, fucose-binding lectins may be particularly important during interactions between the internal defense system of the host

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snail *Biomphalaria glabrata* and sporocysts of the trematode *Schistosoma mansoni*, inasmuch as sporocysts express high levels of glycoproteins bearing the epitope fucosylated LacdiNac (GalNac[Fuca1-3]β1-4GlcNac-R) on their tegument and in their excretory-secretory products (ESPs) (Nyame et al., 2002). Moreover, results of several studies suggest that snail cells and humoral factors bind to fucosylated glycans (reviewed by Yoshino and Coustau, 2011). Specifically, fucoidan, a sulfated polyfucose extracted from brown algae, blocks binding of ESPs from sporocysts of *S. mansoni* to hemocytes of *B. glabrata* (Johnston and Yoshino, 2001) and blocks attachment of *B. glabrata* embryonic (Bge) cells (Castillo and Yoshino, 2002) and hemocytes (Castillo et al., 2007) to the sporocyst tegument. In addition to fucoidan, the monosaccharide mannose 6 phosphate and the sulfated polysaccharides heparin, dextran sulfate, and carrageenan also inhibit attachment of Bge cells to sporocysts (Castillo and Yoshino, 2002). Monoclonal antibody against fucosylated LacdiNac, but not against LacdiNac, reduces Bge cell adhesion to sporocysts, and fucoidan-binding proteins extracted from Bge cells bind to the sporocyst tegument (Castillo et al., 2007). Fibrinogen-related proteins (FREPs) in the plasma of *B. glabrata* bind to fucose residues in sporocyst ESPs from a different trematode, *Echinostoma paraensei*, forming a precipitate (Adema et al., 1997). Thus, fucosyl-reactive receptors appear to occur on the surfaces of Bge cells and hemocytes and in the plasma of *B. glabrata*, mediating both cell attachment to sporocysts and binding to their ESPs.

We were interested in possible mitogenic effects of fucoidan and other glycans on the amebocyte-producing organ (APO) of *B. glabrata*, a region of the anterior pericardial wall in which hemocytes are formed (Lie et al., 1975), and which undergoes activation, i.e., hyperplasia, in response to infection with sporocysts of echinostomes, *Ribeiroia marini*, and *S. mansoni* or following injection with ESPs from *E. paraensei* (reviewed by Sullivan et al., 2004). Indirect support for a role of fucosylated glycans in this mitotic response is that freeze-thaw extracts of miracidia, cercariae, and adults of *S. mansoni*, as well as ESPs of sporocysts, stages that all express fucosylated LacdiNac (Nyame et al., 2002), similarly activate the APO following injection into the hemocoel (Sullivan, 2007; Sullivan et al., 2004). Moreover, although a protostome in a different clade (Ecdysozoa) than *B. glabrata* (Lophotrochozoa), the white shrimp *Litopenaeus vannamei* shows increased mitotic division in hematopoietic tissue following immersion in 100–400 mg/L fucoidan for 3h (Kitikiew et al., 2013). On the basis of the above observations, we investigated whether fucoidan, as well as several other sulfated polysaccharides known to inhibit attachment of Bge cells to sporocysts of *S. mansoni*, could stimulate proliferation of hemocyte precursors in the APO. A non-sulfated polymer of glucose, laminarin, was also tested, because in hemocytes of *Lymnaea stagnalis* this polysaccharide activates protein kinase C (PKC) and ERK pathways (Wright et al., 2006), which are also involved in controlling cell division in the APO (Salamat and Sullivan, 2009).

2. Materials and Methods

2.1. Snails

Snails were reared in aerated aquaria containing artificial pond water (APW) and fed Romaine lettuce ad libitum as described previously (Sullivan et al., 2011). For exposure of

snails by injection into the hemocoel, 10–10.5-mm (shell diameter) adult specimens of the Salvador strain of *B. glabrata*, which is resistant to infection with *S. mansoni* (Paraense and Correa, 1963), were used for most experiments. Additionally, because previous studies showed that the schistosome-susceptible NIH albino or M-line strain of *B. glabrata* (Newton, 1955) maintained at the University of San Francisco (USF-M) responded comparatively weakly to 2 mitogenic substances, i.e., *S. mansoni* miracidia freeze-thaw extract and lipopolysaccharide (Sullivan et al., 2004, 2011), USF-M snails were also used for some procedures. For experiments in which snails were exposed to fucoidan by immersion, juvenile Salvador snails measuring 5–6 mm were used.

2.2. Reagents

The following carbohydrates, all from Sigma Aldrich (St Louis, MO) were prepared as 10 mg/ml solutions in deionized H₂O (dH₂O): λ carageenan, dextran sulfate (sodium salt from *Leuconostoc* spp.), fucoidan (crude, from *Fucus vesiculosus*), L-fucose, L-galactose, heparin (sodium salt from porcine intestinal mucosa), and laminarin from *Laminaria digitata*. Carageenan was heated to 50 °C prior to uptake into the injection needle in order to reduce viscosity, and was allowed to cool for several minutes before injection.

2.3. Fucoidan desulfation

Fucoidan from *F. vesiculosus* contains approximately 26% sulfate (Li et al., 2008), and Castillo and Yoshino (2002) showed that the presence of sulfate groups was important for inhibition of Bge cell-sporocyst binding by polysaccharides. In order to assess the role of sulfate groups, a 100-mg sample of fucoidan was subjected to solvolytic desulfation according to the method of Frenette and Weiss (2000). This procedure involved forming a pyridinium salt of fucoidan, which was then dissolved in dimethyl sulfoxide:methanol. The desulfated product was dialyzed against dH₂O, lyophilized and stored at –20 °C.

2.4. Snail injection

Snails were injected through a hole in the shell and into the hemocoel on the left side, anterior to the digestive gland, with 5 μ l of carbohydrate solutions or dH₂O (controls), and then individually incubated in 500 ml APW at 27 °C for 24h, as described previously (Sullivan et al., 2011). Because certain of the injected substances caused the snails to retract into their shells for varying lengths of time, as explained below, colchicine treatment was not employed to arrest mitotic figures at metaphase (Sullivan, 2007), inasmuch as exposure to the colchicine would not have been uniform among treatment groups.

At 24h post-injection (PI), an interval at which a significant mitotic response occurs to injected substances based on previous studies, the pericardial sac was dissected and fixed in 1/3 Bouin's fluid heated to 50 °C. Mitotic figures in the anterior wall of the pericardial sac were counted in Delafield's hematoxylin and eosin-stained paraffin serial sections using a microscope equipped with a 100x oil immersion objective. For each treatment, 10 APOs were examined, except for injections with desulfated fucoidan (n=15).

2.5. Snail immersion

Groups of 5 Salvador snails measuring 5–6 mm were placed individually in 200 µl of either dH₂O or fucoidan at concentrations of 1 or 10 mg/ml for 3h at 23 °C in an uncovered 96-well plate. Snails attempting to escape the liquid during the exposure were gently pushed down with a paintbrush. Following the exposure, snails were briefly rinsed in dH₂O and placed together in 500 ml APW at 27 °C. At 18h after the conclusion of the 3-h exposure, the group of 5 snails was immersed in 4 ml of 0.1% colchicine in a single 35-mm covered plastic Petri dish at 27 °C (Sullivan, 2007), and at 24h post exposure, pericardial sacs from these snails were fixed in 1/3 Bouin's fluid. Colchicine immersion was used in order to more closely follow the experimental procedure of Kitikiew et al. (2013), who injected fucoidan-exposed white shrimp with vinblastine to arrest mitosis. A total of 15 snails in each of the 3 treatment groups was used. Additionally, APOs from 15 untampered snails, removed from their aquaria and immediately dissected, without exposure to colchicine, were examined.

2.6. Statistical analysis

Mean mitotic counts of snails subjected to different treatments were subjected to one-way analysis of variance (ANOVA), and when a statistically significant difference was found, individual means were compared with the Student-Neuman-Keuls test (SNK) using IBM SPSS Statistics software (Armonk, NY). Additionally, the two-tailed Student's *t* test was used to compare means of colchicine-treated and non colchicine-treated (untampered) snails from the immersion exposure, and the means of dH₂O and fucoidan-injected USF-M snails. Probability values of <0.05 were considered statistically significant.

3. Results

As a general observation, although mortality at 24h PI was rare, injections of carbohydrates, especially polysaccharides, at 10 mg/ml appeared toxic, in that snails often rapidly retracted into their shells during the injection, sometimes expelling hemolymph from the headfoot. Most of these snails remained retracted for up to several hours PI, and relatively few consumed any food during the 24h period prior to fixation. Moreover, some snails showed an increased number of cells with pyknotic nuclei in histological sections of the APO. Conversely, snails injected with dH₂O showed minimal if any distress during injection and usually resumed normal activity within 1h.

In contrast, snails immersed in concentrated fucoidan solutions for 3h showed little evidence of toxicity. Specifically, snails immersed in 1 mg/ml fucoidan behaved no differently than those immersed in dH₂O, crawling around the well and occasionally out of the solution. Snails immersed in 10 mg/ml, while displaying decreased motility and fewer escape attempts, still remained attached by their headfoot to the bottom of the wells, and resumed normal locomotory and feeding behavior when returned to APW.

3.1. Snail injection

ANOVA indicated statistically significant differences among means of Salvador snails ($F_{(9,95)}=6.96$; $P=1.18\times 10^{-7}$), which were then compared by SNK. Among the carbohydrates tested, only fucoidan at a concentration of 10 mg/ml induced a statistically significant

increase in mitotic activity (Fig. 1). Considerable variability occurred among fucoidan-injected snails, with counts in individual APOs ranging from 127 to 1053. Fucoidan at 1 mg/ml had no effect, and desulfation abolished mitogenic activity. The average number of mitotic figures in 10 USF-M snails injected with fucoidan was very low ($1.2 \pm \text{SE } 0.3$), with 3 snails showing no dividing cells in the anterior pericardial wall. In fact, counts in 10 snails injected with dH₂O ($12.8 \pm \text{SE } 3.6$) were significantly higher ($P = 0.005$), although still an order of magnitude lower than counts in Salvador snails.

Although we did not sample other organs in fucoidan-injected snails, no increased mitotic activity was observed in heart tissues, free hemocytes in the ventricle, kidney epithelium, mesenchyme-like tissue of the saccular kidney, mantle epithelium, or albumin gland of Salvador snails, all of which were typically included in dissected pericardial sacs. Thus, based on this limited sampling of other cell types, the mitogenic effect of fucoidan appears to be specific for hematopoietic tissue.

3.2. Snail immersion

Mitotic counts in APOs of snails immersed in dH₂O or 1 mg/ml fucoidan and then treated with colchicine were significantly higher than counts in APOs from untampered snails that had not been exposed to colchicine ($P < 0.05$, Student's *t* test) (Fig. 2). Although counts for snails immersed in 10 mg/ml fucoidan were also higher than those for untampered controls, they showed marked variability, ranging from 3 to 1190 mitotic figures/snail, and the difference between the two means was not statistically significant ($P = 0.06$). Finally, among colchicine-treated snails, ANOVA revealed no statistically significant differences between counts in APOs from dH₂O-exposed and fucoidan-exposed groups, indicating that fucoidan was not mitogenic when snails were exposed by immersion.

4. Discussion

Results of this study show that fucoidan elicits increased mitotic activity in cells of the APO in *B. glabrata*. The presence of sulfate groups is essential for certain biological properties of fucoidan (reviewed by Berteau and Mulloy, 2003), and in our study mitogenic activity was lost after desulfation. Although several other sulfated polysaccharides in addition to fucoidan, including carrageenan, dextran sulfate, and heparin, block attachment of Bge cells to sporocysts of *S. mansoni* (Castillo and Yoshino, 2002), none of these substances showed significant mitogenic activity. Similarly, laminarin did not enhance cell division, despite its reported stimulation of intracellular signaling pathways in snail hemocytes (Wright et al., 2006). Finally, L-fucose, which inhibits precipitation of *E. paraensei* ESPs by FREPs (Adema et al, 1997), was inactive.

The apparent toxicity of the injected carbohydrates hypothetically could have had an effect, either inhibitory or stimulatory, on cell division in the APO. For example, possible direct toxicity to cells of the APO itself, as suggested by pyknotic nuclei in some snails, as well as disruption of homeostasis resulting from toxicity to osmoregulatory, nervous, and other systems in the snails, as suggested by their relatively moribund behavior PI, might be expected to adversely affect APO physiology and depress mitotic activity. Perhaps in part for this reason, fucoidan-injected USF-M snails showed significantly less mitotic activity

than dH₂O-injected snails. In the case of Salvador snails injected with fucoidan, mitogenic activity presumably compensated for any potential antimitotic toxic effects. Whether mitogenicity of the other injected substances was masked by their toxicity is not known. Conversely, by causing inflammation or tissue repair involving hemocytes, toxic substances could conceivably stimulate cell division in the APO. However, the other tested substances, several of which showed similar toxic effects, were not mitogenic, suggesting that fucoidan toxicity alone was not the cause of elevated mitotic activity.

Unlike results in white shrimp (Kitikiew et al., 2013), immersion in 1 mg/ml or 10 mg/ml fucoidan did not stimulate cell division in the APO. The epithelium covering the headfoot and mantle and lining the digestive tract of *B. glabrata* consists of only a single layer of cuboidal to columnar cells (Pan, 1958), and is obviously permeable to certain small lipophilic organic molecules, e.g., colchicine. However, fucoidan, probably due to its high molecular weight (20,000–200,000 D, according to product documentation) and negative charge, is unable to diffuse across the external or gut epithelia in sufficient concentration to stimulate the APO. In view of the phylogenetic distance separating arthropods and molluscs, it is unknown whether the uptake of and mitotic response to fucoidan occurs by evolutionarily conserved processes in both phyla.

Fucoidan has diverse effects in vertebrates both in vivo and in vitro (Berteau and Mulloy, 2003; Lie et al. 2008), and while the possible relevance of such effects to invertebrates must be interpreted with caution, it is nonetheless interesting that fucoidan actually inhibits cell division in mammalian cancer and smooth muscle cells (reviewed by Li et al., 2008). Thus, the mitogenicity of fucoidan in *L. vannamei* and *B. glabrata* is a previously unreported bioactivity of this substance.

As reported in previous studies, the anterior pericardial wall of USF-M snails is relatively unresponsive to injected schistosome miracidia extracts and lipopolysaccharide, both of which trigger increased cell division in the APO of Salvador snails (Sullivan et al., 2004, 2011). USF-M adults have few (and in some specimens no) discernable follicles of hematopoietic tissue in the anterior pericardial wall, and among these mitotic figures rarely are seen. Not unexpectedly then, no mitotic response to fucoidan was observed in these snails. Preliminary studies indicate that the atrophic condition of the APO may be a trait specific to USF-M snails (unpublished), and heritability of this trait is currently being investigated.

The manner by which fucoidan elicits cell proliferation in the APO, i.e., whether by directly stimulating hemocyte precursors or by first interacting with cells or plasma factors external to the APO, remains to be determined. However, results of this study provide support for the hypothesis that fucosylated glycans on the tegument and in the ESPs of larval schistosomes are in part responsible for triggering cell division in the APO of snails infected with these trematodes or injected with their extracts.

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Highlights

- Fucoidan is mitogenic for snail hematopoietic tissue.
- Presence of sulfate groups is necessary for mitogenic activity of fucoidan.
- Sulfated polysaccharides other than fucoidan are not mitogenic.
- Immersion in concentrated fucoidan solutions does not stimulate mitosis.
- Fucosylated glycans from schistosome sporocysts may elicit cell proliferation.

Figure 1.

Average number, $\pm$ standard error, of mitotic figures in the amebocyte-producing organ of adult *Salvador Biomphalaria glabrata* 24h post injection with 5 μ l of deionized water (dH₂O), heparin sulfate (HEP), dextran sulfate (DEX), λ carrageenan (CAR), fucoidan (FCN), desulfated fucoidan (dFCN), L-fucose (FUC) or L-galactose (GAL), all at a concentration of 10 mg/ml except for FCN-1, (=1 mg/ml). n=10 for each group, except for dFCN (n=15). Differences between bars marked by the same letters are not significantly different ($P > 0.05$, Student-Neuman-Keuls test).

Figure 2.

Average number, $\pm$ standard error, of mitotic figures in the amebocyte-producing organ of juvenile *Salvador Biomphalaria glabrata* 24h following a 3-h immersion in 200 μ l of deionized water (dH₂O), 1 mg/ml fucoidan (FCN-1) or 10 mg/ml fucoidan (FCN). Snails were incubated in 0.1% colchicine for 6h prior to fixation. Untampered snails were not immersed for 3h or exposed to colchicine. n=15. Asterisks denote means that are significantly greater than that of the untampered group ($P < 0.05$, Student's t test).