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Immunohistochemical Localization of a 27 KDa Protein in the Plerocercoids of *Spirometra erinacei*

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Abstract

A 27 kDa protein of *Spirometra erinacei* plerocercoids can be recognized specifically by a murine monoclonal antibody produced against the 27.5 kDa protein of *S. mansonoides* plerocercoids. Using this monoclonal antibody, localization of the 27 kDa protein in the plerocercoid was examined immunohistochemically by strepto-avidin-biotin method with emphasis on the expression of the protein in the plerocercoid during the shedding process in mouse intestines. We observed that the 27 kDa protein was mainly present on the surface of the tegument and in the subtegumental cells of plerocercoids. This molecule was concentratively expressed at the border of the shedding site, and it was expressed in the neck until shedding began.

Key words: Immunolocalization, proteinase, *Spirometra erinacei*, plerocercoid, 27 kDa protein

Introduction

Plerocercoids of some species of the genus *Spirometra* produce a substance having growth hormone-like activity (Mueller, 1963; Phares and Hirai, 1990; Odening and Bockhardt, 1982). In the case of *S. erinacei*, a 27 kDa protein produced by plerocercoids displaces [^{125}I] labeled human growth hormone from its hepatocyte membrane receptors prepared from a pregnant rabbit. The purified 27 kDa protein stimulates significantly the [^{3}H]thymidine incorporation into DNA of primary cultured mouse hepatocytes, and the amino acid sequence of its cleavage fragment shares a 66.7% homology with that of cathepsin L (Hirai *et al.*, 1990). In addition, the hepatocyte proliferation activity of the 27 kDa protein can be inhibited by both pepstatin and leupeptin (Tsuboi *et al.*, 1992). Cathepsin L, a proteinase, stimulates the proliferation of liver tissues (Terayama *et al.*, 1985). On the other hand, some reports indicated that a number of proteinases may help parasites to survive in their hosts or to complete their life cycle (Zerda *et al.*, 1988; Rosenthal *et al.*, 1988; Carmona *et al.*, 1993). Therefore, the 27 kDa protein may be a multipotent molecule involved in the host-parasite relationship. However, little is known about its synthesizing site, except for some speculations.

The purpose of this study is to determine the localization of the 27 kDa protein in the plerocercoid and its expression during the process of shedding off the body.

Materials and Methods

Plerocercoids

Plerocercoids of *S. erinacei* were collected from two species of snakes, *Elaphe quadrivirgata* and *Rhabdophis tigrinus*, captured in the eastern parts of Shimane Prefecture, Japan, and stored in the subcutaneous tissues of golden hamsters.

Animals

Male ICR mice, 8 weeks old, were purchased from Japan Clea Co. (Tokyo, Japan). Several

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plerocercoids were orally injected into the stomachs of mice under light anesthesia. Forty minutes after oral administration, the mice were sacrificed to recover the plerocercoids with or without shedding from their intestines.

Reagents

The Histofine strepto-avidin-biotin (SAB) (M) kit used for immunolocalization was purchased from Biochemical Industries Ltd (Tokyo, Japan). A kit for immunoblotting was bought from Vector Laboratories (VECTASTAIN, Burlingame, CA, USA). The murine-anti-27.5 kDa protein monoclonal antibody (MAb, C3-2.2) was prepared according to published methods (Köhler and Milstein, 1975; Nowinski *et al.*, 1979) by the University of Nebraska Medical Center Monoclonal Antibody Facility.

Immunoblotting analysis

Plerocercoids removed from the golden hamsters were washed several times in physiological saline solution and an extract was made by a modified method of the procedure described by Phares (1988) as follows. Plerocercoids were homogenized in 25 mM Tris-HCl, pH 7.6, with 150 mM NaCl in a ratio of 1g of worm/5 ml of buffer. The homogenate was centrifuged at 12×g for 5 min. The pelleted calcareous corpuscles were discarded, and the supernatant was centrifuged at 5000×g for 20 min at 4°C. The pellet containing crude membrane was dispersed in 25 mM Tris-HCl, pH 7.6, with 1% Triton X-100 (1 ml of solubilizing buffer/g of original worm), then solubilized for 30 min on ice with gentle sterring. The mixture was centrifuged at 105,000×g for 90 min at 4°C. The supernatant was added to Biobeads SM-2 (Bio-Rad Lab. Co. USA) in a ratio of 15 mg of beads/ml of solubilized solution, and gently mixed for 90 min on ice to remove the Triton X-100 from the solubilized material. The solubilized material was separated from the Biobeads by filtration over a glass wool filter.

The solubilized material was then separated by sodium dodecyl sulphate and polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gel as described by Laemmli (1970). The separated substance was electrophoretically transferred to a polyvinylidine difluoride (PVDF) protein sequencing membrane (Bio-Rad Co. LTD, USA) by means of the semidry method (Towbin *et al.*, 1979) using a NOVABLOT Electro Transfer Kit (Pharmacia LKB Biotechnology Co., Sweden). After transfer, the PVDF membrane was washed sufficiently, then incubated with 5% skim milk in Tris-buffered saline containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20 (TBST) for 1 h. After being washed 3 times in TBST, the membrane was subjected to reaction with murine-anti-27.5 kDa protein MAb at a dilution of 1:500 for 2 h. After further washing 5 times, the membrane was incubated with the biotinylated rabbit anti-mouse IgG for 30 min. After repeated washing as above, the membrane was finally immersed in alkaline phosphatase conjugated avidin-biotin conjugate solution for another 30 min. After a final washing of 5 times in Tris-buffered saline containing 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl, the antigen-antibody binding was visualized by adding a substrate solution containing 0.2 mg/ml of nitroblue tetrazolium chloride, 0.15 mg/ml of 5 bromo-4 chloro-3 indolyl phosphate, 0.1 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris-HCl, pH 9.5. Another gel run at the same time was stained with Coomassie Brilliant Blue. All procedures were done at room temperature unless otherwise indicated.

Paraffin embedding strepto-avidin-biotin method

Plerocercoids were washed sufficiently in phosphate-buffered saline (PBS: 50 mM sodium phosphate, 150 mM NaCl, pH 7.4), and fixed in 10% phosphate-buffered formalin (pH 7.2) for 7 h. They were embedded in low melting point-paraffin, never exceeding 58°C. The specimens were cut into 5 μm thick sections, and mounted on poly-L-lysine-coated slides.

They were deparaffinized in a series of graded ethanol, and washed with PBS for 10 min to remove the residual ethanol. To block endogenous peroxidase activity, the sections were immersed in 3% hydrogen peroxide solution for 15 min and washed three times with PBS. The sections were then incubated with PBS containing non-immune rabbit serum (1:10 dilution) for 10 min to block nonspecific binding sites. Subsequently, the sections were incubated with murine-anti-27.5 kDa protein MAb (1:24,000 dilution of ascites fluid) at 4°C overnight.

They were washed three times with PBS, then incubated with biotinylated secondary antibody for 10 min. After washing, the bound immunoglobulin was visualized by incubating the sections with peroxidase-conjugated strepto-avidin for 5 min. The color was developed with Tris-HCl buffered solution, pH 7.4, containing 0.01% hydrogen peroxide and 0.05% 3,3'-diaminobenzidine substrate. Finally, the sections were counterstained with water-based hematoxylin (Merck, USA). All procedures were done at room temperature unless otherwise indicated. For negative control, PBS and normal mouse serum took the place of MAb.

Results

Identification of the specificity of the MAb

Using the immunoblot analysis, the 27 kDa protein was specifically recognized by the MAb produced against the 27.5 kDa protein derived from plerocercoids of *S. mansonoides*. No other proteins in the worm crude extract cross-reacted with this MAb (Fig. 1).

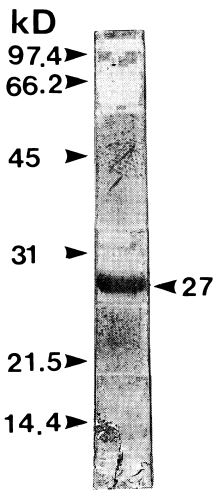

Fig. 1 Immunoblotting analysis of worm extract with anti-27.5 kDa protein monoclonal antibody.

Immunohistochemical localization of the 27 kDa protein in the plerocercoids of S. erinacei

Immunolocalization of the 27 kDa protein in the plerocercoids of *S. erinacei* was examined with the anti-27.5 kDa protein MAb. The immunoreactivity of this antibody was concentratively recognized on the surface of the tegument and in the subtegumental cells of the plerocercoid (Fig. 2-2). No difference in staining intensity was found between the scolex and the body. The staining was not seen when Mab was omitted (Fig. 2-1).

Expression of the 27 kDa protein at the site of shedding

In addition to the strong staining of the tegumental surface and subtegumental cells, the rear part of the neck were deeply stained after shedding off the body, while the contiguous tissue was lightly stained (Fig. 2-4). A relatively deep staining was recognized at the border of the shedding site (Fig. 2-5). The staining was not seen when MAb was omitted (Fig. 2-3).

Discussion

A number of research works have been done on the biological activities of the growth factor produced by plerocercoids of some species of the genus *Spirometra* (Hirai *et al.*, 1993; Phares, 1987; Phares and Hirai, 1990). Recently, Hirai *et al.* (1990) purified a 27 kDa protein from *S. erinacei* plerocercoids, and showed that the 27 kDa protein binds to an anti-human growth hormone MAb as in the case of the 27.5 kDa protein from *S. mansonoides* (Phares *et al.*, 1988). Furthermore, partial amino acid sequences of the 27 kDa protein have parts highly homologous to the 27.5 kDa protein (Phares, personal communica-

Fig. 2. Immunostaining of the 27 kDa protein. Bars represent 50 μm.
Fig. 2-1. Control, treated with PBS or normal mouse serum, being not stained (body part).
Fig. 2-2. Immunolocalization of 27 kDa protein in plerocercoids, showing distinct staining on surface (arrow "↓") and subtegumental cells of the body part (arrow "↑").
Fig. 2-3. Control for shedding experiment. Treated identically as "Fig. 2-1", being not stained (neck part).
Fig. 2-4. Expression of 27 kDa protein at shedding site of the neck part, stained deeply (arrow).
Fig. 2-5. Expression of 27 kDa protein at shedding site of the neck, stained lightly (arrow).

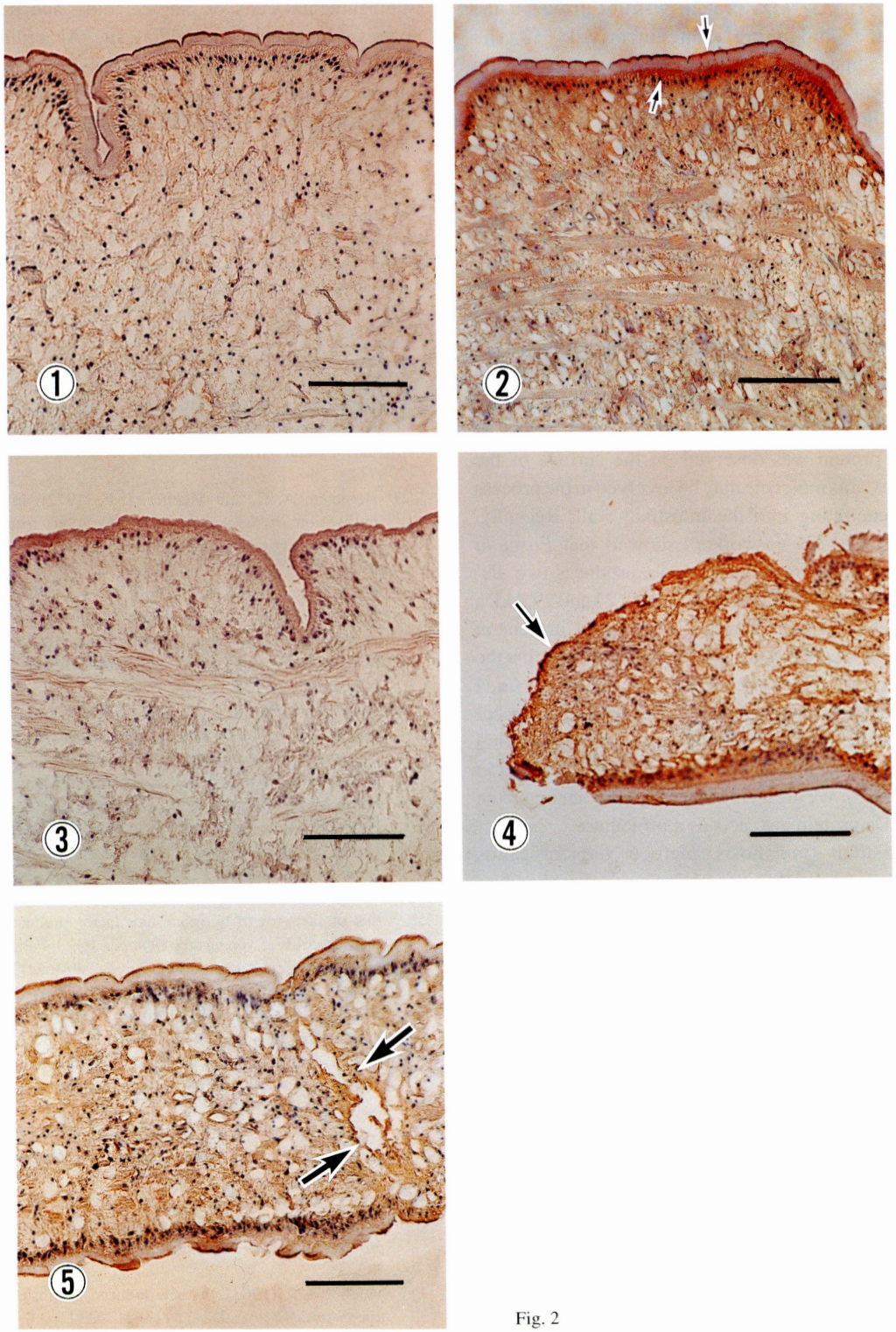

Fig. 2

tion). The 27 kDa protein was localized on the surface and more concentratively in the subtegumental cells, suggesting that this protein was synthesized by subtegumental cells and spreaded over the microtriches and the surface membrane. The reason why the tegument itself was negative is unknown. Kim *et al.* (1992) showed that a 29 kDa protein derived from a Korean strain of *Spirometra* was likely to be synthesized in subtegumental cells of the plerocercoid. Further study is necessary to compare our 27 kDa protein with the 29 kDa protein from the Korean strain. Tsuboi *et al.* (1992) showed that the hepatocyte growth activity of the purified 27 kDa protein can be inhibited by both pepstatin and leupeptin, so that this protein seems to have a cysteine proteinase-like substance activity. Because the 27 kDa protein was observed on the surface of the scolex, this molecule may be involved in the process of penetrating into the intestinal wall. Recently, Smith and his co-workers showed that *Fasciola hepatica*, a parasitic trematode, produces two distinct subclasses of cathepsin L with 27 and 29.5 kDa molecular weight (Smith *et al.*, 1993; Carmona *et al.*, 1993; Dowd *et al.*, 1994). It is of interest that the cathepsin L proteinase secreted by *Fasciola hepatica* prevents antibody-mediated eosinophil attachment to newly excysted juveniles *in vitro* by cleaving immunoglobulin at the hinge region (Carmona *et al.*, 1993). We are trying to clarify whether or not the 27 kDa protein also acts as a proteinase.

In their final hosts, plerocercoids of genus *Spirometra* develop into adult worms after shedding off their "tails" piece by piece, while in their second intermediate hosts, such as the mouse, plerocercoids attach to the mucous membranes of the intestines, and, after shedding off their body, only the scolex penetrates into the gut wall. On the other hand, Lev *et al.* (1986) reported a novel mechanism of host-parasite interaction that a lectin in *Giardia lamblia* was activated by secretions from the human duodenum. Related to this, Berntsen and Mueller (1964; 1972) demonstrated that both bile and trypsin are essential for the occurrence of shedding in plerocercoids. Further research is needed to formulate the mechanisms of the expression of the 27 kDa protein.

In conclusion, we found that the 27 kDa protein localized on the surface of the tegument and in the subtegumental cells of plerocercoids, and it was expressed at the body-shedding site. Accordingly, we suggest that the subtegumental cells of *S. erinacei* plerocercoids may synthesize the 27 kDa protein while taking part in the body-shedding process.

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