SELECTIVE IMMUNOSTAINING OF MEHLIS' GLAND OF *OPISTHORCHIS VIVERRINI* BY ANTIBODY AGAINST RAT DIACYLGLYCEROL KINASE

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Abstract. The Mehlis' gland of *Opisthorchis viverrini* was selectively and intensely immunopositive with an antibody against rat diacylglycerol kinase γ, and its entire structure with associated radiating processes was clearly demonstrated by immuno-light microscopy. In immuno-electron microscopy, the immunopositive processes were revealed to contain many vesicles and vacuoles and the immunoreactive materials were deposited diffusely in the cytoplasm except for the vesicular interior. The present findings suggest that diacylglycerol kinase is present and plays roles in PKC (protein kinase C)-related signaling in the Mehlis' gland of *O. viverrini*. This further suggests the possibility of a new way to protect from the infection of *O. viverrini* in humans by using diacylglycerol kinase as a therapeutic target.

Keywords: *Opisthorchis viverrini*, Mehlis' gland, diacylglycerol kinase γ, immunohistochemistry

INTRODUCTION

The human liver fluke, *Opisthorchis viverrini*, is a major risk factor for cholangiocarcinoma (CCA) in Thailand and other Southeast Asian countries (Sriamporn *et al*, 2004; Shin *et al*, 2010). The molecular mechanisms underlying *O. viverrini*-induced CCA are thought to be multifactorial, and the pathogenesis of cell-transformation in the bile duct epithelium leading to CCA may be mainly from the physical irritation of the *O. viverrini* suckers on the bile duct epithelium and immunopathological factors (Sripa *et al*, 2007; 2008).

Among the worm systems that might be targeted for therapeutic intervention, the reproductive system is an important candidate. This system is metabolically very active in adult worms and any disruption caused to it will inhibit reproduction. It is thus essential to clarify the anatomy of the gonads and reproductive

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systems in *O. viverrini*, in both light and electron microscopy. Present information in this regard is still insufficient as compared with their mammalian counterparts. The female reproductive system of *O. viverrini* is composed of a single ovary with an oviduct, a seminal receptacle, a pair of vitelline glands with ducts, and the Mehlis' gland surrounding the ootype and secreting into it lubricants and substances associated with the shell production (Smyth, 1962; Nithikathkul *et al*, 2007; Khampoosa *et al*, 2012).

For individual organs to be thoroughly analysed, especially in morphology, it would be advantageous to obtain immunohistochemical markers for each of them. During a pilot attempt to look for immunohistochemical markers specific to individual organs of the *O. viverrini* reproductive system, it was found that the Mehlis' gland was selectively immunostained by an antibody against the γ isozyme of rat diacylglycerol (DAG) kinase, implying the presence of a very similar enzyme in *O. vivverini*. DAG kinase is known to be involved in conversion of DAG to phosphatidate, which along with the DAG kinase are second messengers in PKC (protein kinase C)-related lipidsignaling (Kanoh *et al*, 1990; 2002; Goto *et al*, 2008). It is therefore possible that DAG kinase is critical in the lipid signaling of *O. viverrini* and that, given its concentration in the Mehlis' gland, it is vital to the production of eggs. The immunohistochemical features of the Mehlis' gland of *O. viverrini* are described for the first time in this study.

MATERIALS AND METHODS

Metacercariae of *O. viverrini* were collected from naturally infected cyprinid fish and were then used to infect Syrian golden hamsters by stomach intubation and adult flukes harvested four weeks later. All procedures were conducted in accordance with Guidelines for the Care and Use of Laboratory Animals at Khon Kaen University. This study was approved by the Animal Ethics Committee of the Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Reference No.0514.1.12.2/70). The harvested flukes were fixed with 4% paraformaldehyde/0.1 M phosphate buffer for two hours. Specimens were dipped into 30% sucrose/0.1 M phosphate buffer and then cryosections of 20 μm thickness were cut. The sections were then permeabilized with 0.1%TritonX-100/PBS, incubated with 0.3% H_2O_2/methanol, and then 10% normal goat serum/PBS for 30 minutes. The sections were incubated with anti-rat DAG kinase γ rabbit IgG (2 μg/ml) for 24 hours. The specificity of the antibody was fully reported previously by two (YH & KG) of the present authors (Nakano *et al*, 2012). The sections were subsequently incubated with biotinylated anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA) diluted at 1:200 for the DAB (diaminobenzidine) reaction using the VECTASTAIN Elite ABC kit (Vector Laboratories). After the DABreaction, some of the sections were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer for 1 hour and further with 1% OsO_4 in phosphate buffer for 1 hour. After *en-bloc* staining with uranyl acetate solution, the sections were embedded in Epon at 60°C for 1 day. Ultrathin sections without lead staining were observed in a Jeol 1010 electron microscope. Some of the sections, after incubation with the DGK antibody, were further incubated with FITC-conjugated rabbit IgG (Sigma-Aldrich, St. Louis, MO) and observed for
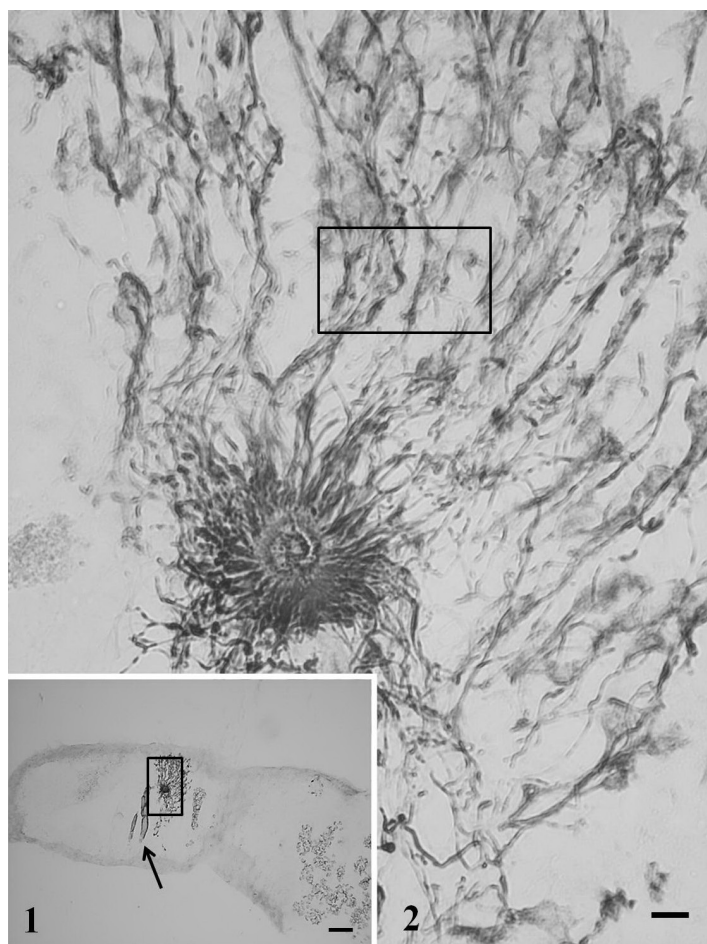
RESULTS

Using immuno-light microscopy (Figs 1 and 2), distinct immunoreactivity for rat DAG kinase γ was detected in the Mehlis' gland, which was shown to consist of a central cell-mass surrounding the ootype. Densely staining processes radiating from the Mehlis' gland were clearly recognizable. In contrast, there was no immunoreactivity elsewhere in the worm. Although the surface of elongated structures close to the Mehlis' gland appeared to be positive in immuno-DAB-light microscopy (Fig 1), no significant immuno-fluorescence was detected in confocal laser microscopy in structures corresponding to those in immuno- DAB-light microscopy (Figs 3a and 3b). At higher magnification in immuno-DAB- and immuno–fluorescence-light DAB-microscopy (Figs 2 and 3c), varicose processes were discerned to be intensely immunopositive.

In immuno-DAB-electron microscopy, aggregations of cells having polygonal shapes and radiating cell processes with variable thicknesses of 0.1~0.7 μm were immunopositive, and the immunoreactive processes contained numerous vesicles and vacuoles of varying diameters (150~400 nm) (Fig 4a and 4b). The immunoreactive materials were
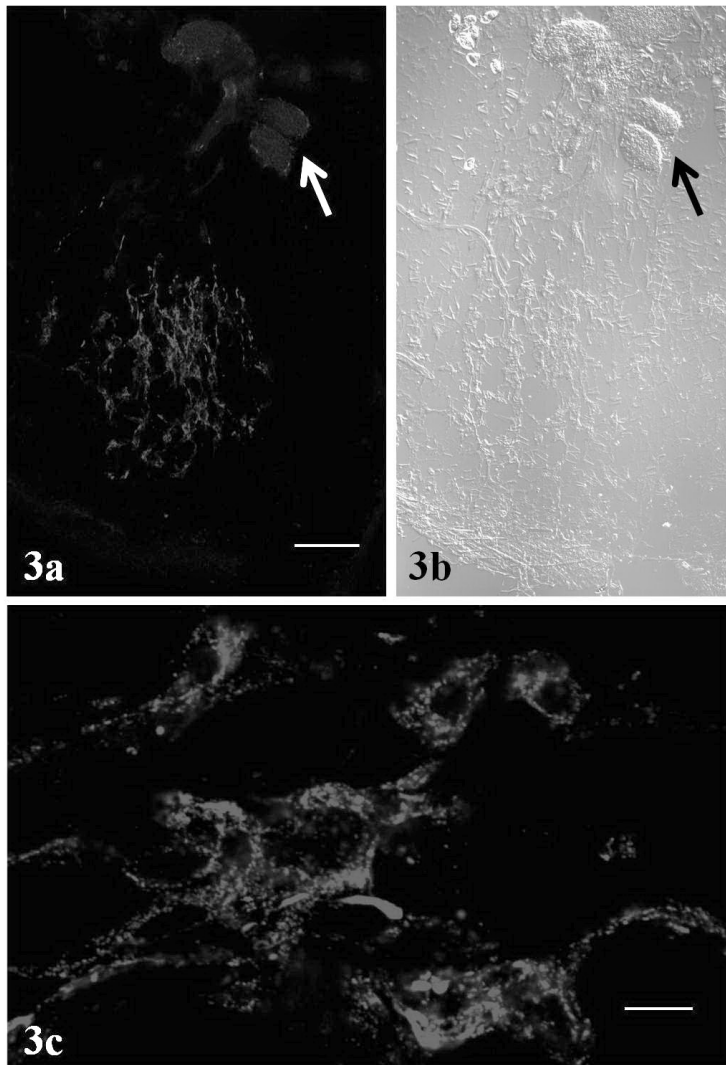

Fig 1–Immuno-DAB-light micrograph of coronal section of *O. viverrini* with antibody against rat DAG kinase. Note selectively and intensely immunostained Mehlis' gland in the posterior half of the worm. The oval structures indicated by arrow are eventually interpreted as pseudo-reactive in Fig 3. Bar represents 100 μm.

Fig 2–Higher magnification micrograph of a portion of radiating processes of Mehlis' gland enclosed by a rectangle in Fig 1. Bar represents 10 μm.

immunofluorescence in confocal laser microscopy (Nikon, Tokyo, Japan).

In control experiments, the primary antibody alone was omitted in the process of treating sections for immunohistochemistry.

Fig 3–Immuno-fluorescence confocal laser micrograph (3a) with the same antibody of a portion corresponding to that in Fig 2 and its higher magnification micrograph (3c), and differential interference contrast (DIC) image (3b) of the same portion as shown in 3a by confocal laser microscopy. Note the absence of significant immunofluorescence in the oval structures (arrows) adjacent to the Mehlis' gland, which are observed in Fig 1. Bars represent 100 μm (3a, 3b) and 10 μm (3c).

deposited diffusely in the cytoplasm, but not within the vesicular membranous structures. The immunopositive processes were associated with thinly extended but immunonegative cellular processes with distinct interstitial spaces intervening.

DISCUSSION

An immunoreactive reagent, such as described here, provides a means of further characterization of a bio-structural element using light and electron microscopy. The other issue is the functional significance of the molecule in the structural element. With regard to the first issue in this case, no histological marker specific to the Mehlis' gland has so far been available, resulting in high difficulty to specify the gland in section efficiently and reliably for electron microscopic examination at present. In this case, further information of the structural details of the Mehlis' gland and its spatial relationships to adjacent reproductive and non-reproductive organs of *O. viverrini* has been gained. There has been a study demonstrating that an antibody against an antigen isolated from the tegument of *Fasciola* reacts with various organs including the Mehlis' gland cells (Anuracpreeda *et al*, 2006). However, different from the present DGK antibody, no detailed information on the identification of the antigen and because of its immunoreaction not specific to Mehlis' gland, but to various organs,
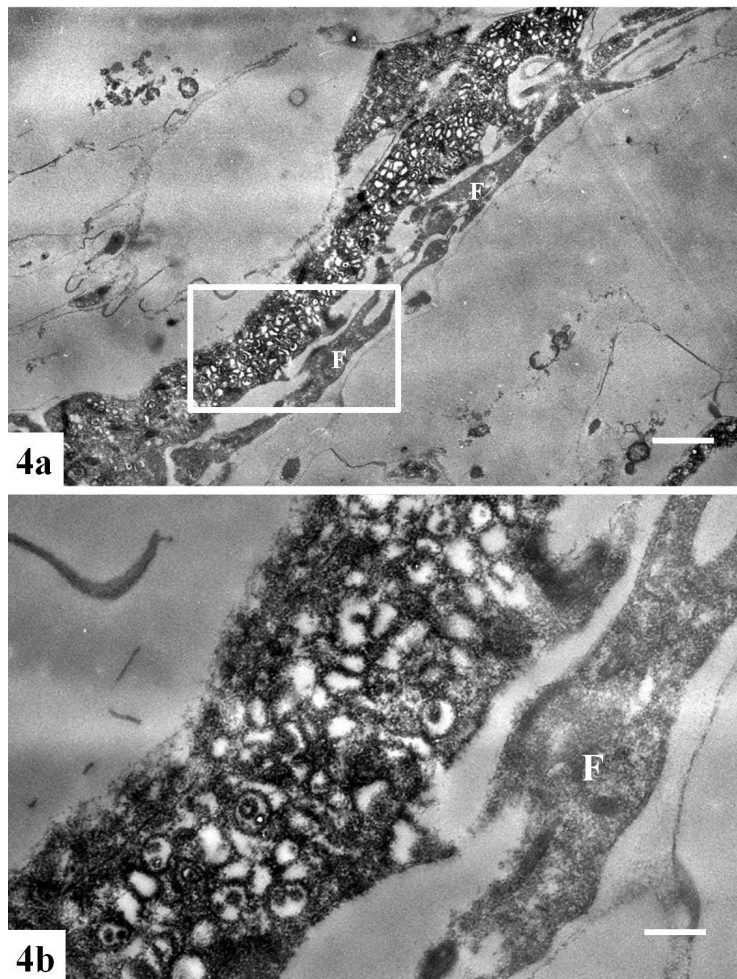
Fig 4–Immuno-DAB-electron micrographs (4a) of immunopositive cell processes, and a higher magnification micrograph (4b) of a portion enclosed by the rectangle in 4a. Note numerous vesicles and vacuoles in the process and immunoreactive materials are deposited diffusely in the cytoplasm except for the vesicular interiors. F: immunonegative cell associated with the immunopositive one with a wide interstitial space intervening. Bar represents 2 μm (Fig 4a) and 500 nm (Fig 4b).

antigen immunoreactive with the present antibody against rat DAG kinase γ can be characterized. It is possible that the antigen represents an authentic DAG kinase of *O. viverrini*. In the PI signaling, DAG is the first identified mammalian second messenger to enhance the activity of protein kinase C (PKC) and it is converted to phosphatidate by DAG kinase. Therefore, DAG kinase is a key regulator of PKC activity by its role as an attenuator of DAG (Kanoh *et al*, 1990, 2002; Goto *et al*, 2008).

Considering the plausible functions of the Mehlis' gland such as lubrication of the uterus for the passage of eggs, release of shell globules from the vitelline cells as well as activation of spermatozoa (Smyth, 1962), the present findings suggest that DAG kinase plays a role in reproduction of *O. viverrini*, possibly via regulation of DAG-activating PKC activity during the maturation of the eggs and sperm. If verified, this could lead to a new way to limit the impacts of *O. viverrini* on humans by using DAG kinase as a therapeutic target.

the present antigen is regarded as much more useful as an immunohistochemical marker for the Mehlis' gland to obtain much more information on its ultrastructural studies.

With regard to the second issue to be considered, western blotting, the parasite

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