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Monoclonal antibodies against 27.8 kDa protein receptor efficiently block lymphocystis disease virus infection in flounder *Paralichthys olivaceus* gill cells

Xiu-Zhen Sheng, Mu Wang, Jing Xing, Wen-Bin Zhan*

Laboratory of Pathology and Immunology of Aquatic Animals, Ocean University of China, 5 Yushan Road, Qingdao 266003, PR China

ABSTRACT: In previous research using co-immunoprecipitation, a 27.8 kDa protein in flounder *Paralichthys olivaceus* gill (FG) cells was found to bind lymphocystis disease virus (LCDV). In this paper, 13 hybridomas secreting monoclonal antibodies (MAbs) against the 27.8 kDa protein were obtained, and 2 MAbs designated as 2G11 and 3D9 were cloned by limiting dilution. Analyzed by indirect enzyme-linked immunosorbent assay (ELISA) and western blotting, the MAbs specifically reacted with the 27.8 kDa protein of FG cells. Confocal fluorescence microscopy and immunogold electron microscopy (IEM) provided evidence that the epitopes recognized by these MAbs were located primarily on the cell membrane and occasionally in the cytoplasm near the cell membrane of FG cells. The MAbs could block LCDV binding after MAbs were pre-incubated with isolated membrane proteins of FG cells in a blocking ELISA, and MAbs also could inhibit LCDV infection of FG cells in culture. Moreover, several target tissues of LCDV in flounder, including gill, stomach, intestine and liver, displayed the presence of the LCDV receptor-27.8 kDa. These results strongly supported the possibility that the 27.8 kDa protein is the putative receptor specific for LCDV infection of FG cells in flounder.

KEY WORDS: MAbs · Virus–host cell interactions · Co-immunoprecipitation · ELISA · Western blotting · Immunogold electron microscopy · Confocal fluorescence microscopy

INTRODUCTION

Lymphocystis disease virus (LCDV), a large icosahedral DNA virus classified in the iridovirus family, is the causative agent of lymphocystis disease, which occurs in marine and freshwater fish species and is characterized by formation of papilloma-like lesions on the body surface and sometimes in the internal tissues of fish (Brown 1986, Sarasquete et al. 1998, Sheng & Zhan 2004). Ultrastructural analyses based on the LCDV infection of flounder *Paralichthys olivaceus* gill (FG) cells, a cell line derived from gill tissue of flounder (Tong et al. 1997), demonstrated viral attachment to the cell surface and entry by receptor-mediated endocytosis (Lv et al. 2003). Understanding how viral proteins and host cell receptors mediate this initial interaction is instrumental for elucidating the pathogenesis of viral infections.

Virus receptor research in aquatic animals has been limited, but some current information is available using protein–protein interaction methods to investigate virus receptor interactions. A 250 kDa protein in several fish cell lines was shown to bind marine birnavirus (MABV) by virus overlay protein

*Corresponding author. Email: wbzhan@ouc.edu.cn

binding assay (VOPBA) (Imajoh et al. 2003), white VP26 protein of white spot syndrome virus (WSSV) binding to actin of shrimp was demonstrated using co-immunoprecipitation (Xie & Yang 2005), and monoclonal antibodies (MAbs) generated against rainbow trout *Oncorhynchus mykiss* gonad cells (RTG-2) have been used to protect cells from infection by viral haemorrhagic septicemia virus (VHSV) (Bearzotti et al. 1999).

In previous research, LCDV was found to bind a 27.8 kDa protein in FG cells using co-immunoprecipitation, and the virus binding was partially blocked by the polyclonal anti-27.8 kDa protein antiserum. However, western blotting demonstrated the polyclonal anti-27.8 kDa protein antiserum had weak cross-reactivity with the 42.7 kDa protein of FG cells, which suggested the 2 proteins shared some common epitopes (Wang et al. 2011). Thus, in the present study, MAbs were produced to the 27.8 KDa receptor in FG cells in order to investigate the receptor distribution in flounder tissue and the inhibitory effect of cultured FG cells on infection by LCDV.

MATERIALS AND METHODS

Cell culture and virus purification

Monolayers of flounder gill cell line FG were grown at 22°C with 2% CO_2 in Eagle's MEM (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. The FBS was reduced to 2% in maintenance medium following LCDV inoculation of the cultures.

Flounder with lymphocystis nodules on their body surface were obtained from a farm located in Qingdao, Shandong province of China. LCDV particles were isolated and purified according to the methods described by Cheng et al. (2006).

27.8 kDa receptor protein purification

The cell membrane proteins of FG cells were prepared as described by Wang et al. (2011). After the concentrations of proteins were determined by the Bradford method (Bradford 1976), they were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the 27.8 kDa band was cut out and electroeluted overnight at 25 V in an electroelutor (Sub-Cell, Bio-Rad). After dialysis in distilled water (DW) overnight, the protein was concentrated using a freeze dryer (PowerDry LL3000, Heto-holten) and resuspended in phosphate buffered saline (PBS). The purified 27.8 kDa protein concentration was then quantified (Bradford 1976).

MAb production

MAbs were produced by immunization of BALB/c mice with purified 27.8 kDa protein. Myeloma cells (P3-X63-Ag8U1, P3U1) in log phase of growth were fused with spleen cells, and hybridomas were produced by the method described previously (Cheng et al. 2006). The supernatants from wells with growing hybridomas were screened using indirect enzyme-linked immunosorbent assay (ELISA). Positive cultures were amplified, cloned and sub-cloned using the limiting dilution method, and further screened by western blotting in addition to ELISA. All studies were conducted in accordance with institutional, national and international guidelines concerning the use of animals in research.

MAbs screening by ELISA was performed according to Cheng et al. (2006). Flat-bottom 96-well microplates (Costar) were coated with the 27.8 kDa proteins (2 µg $well^{-1}$) and then blocked with 3% bovine serum albumin (BSA). The hybridoma culture fluids were added as primary antibody and alkaline phosphatase (AP)-conjugated goat-anti-mouse immunoglobin (Ig) (Sigma) diluted 1:4000 as secondary antibody. Then the chromogenic assay with *p*-nitrophenyl phosphate (*p*NPP) as substrate was performed in microplates, and absorbencies were measured with an automatic ELISA reader at 405 nm (Molecular Devices). Mouse antiserum and P3U1 culture fluids were used as positive and negative controls, respectively.

For western blotting (Wang et al. 2011), FG cell membrane proteins were run on 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with skim milk, hybridoma culture fluids were incubated as the primary antibody, and AP-conjugated goat-anti-mouse Ig as the secondary antibody. Color was developed with substrate solution containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium (BCIP). The reaction was stopped by washing in DW to remove excess substrate when the positive bands were visualized.

The Mabs were purified from hybridoma culture fluids by $(NH_4)_2SO_4$ precipitation and protein A-Sepharose chromatography according to Lee-Hsu et al. (1988), and the titers of purified MAbs were determined by ELISA.

Blocking ELISA (B-ELISA)

Ninety-six wells were coated with the membrane proteins of FG cells (50 µg well^{-1}) in 100 µl PBS, incubated overnight at 4°C, washed 3 times with PBS (pH 7.4) containing 0.05% Tween-20 (PBST) and blocked with 200 µl of 3% BSA in PBS for 1 h at 37°C. The microplate was washed as above and 100 µl anti-27.8 kDa MAbs (0.01, 0.1, 1, 5 and 10 µg well^{-1}) were added as the blocking antibody. After incubation for 1 h at 37°C, the microplate was washed, 50 µl of purified LCDV (10 µg well^{-1}) in PBS was added, and the plate was incubated for 3 h at 22°C. Following further washings, 100 µl of rabbit anti-LCDV serum (Wang et al. 2011) was added and incubated for 1 h at 37°C. The microplate was washed and 100 µl AP-conjugated goat-anti-rabbit Ig (Sigma) diluted 1:1000 in PBS was added and incubated for 1 h at 37°C. After the last wash, 100 µl 0.1% (w/v) *p*NPP in carbonate-bicarbonate buffer containing 0.5 mM $MgCl_2$ was added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped with 50 µl of 2 M NaOH well^{-1} and absorbance was measured with an automatic ELISA reader at 405 nm. Incubation with MAb 1D5 against WSSV (Zhan et al. 1999), which had no positive reaction with FG cell, and an isotype matched with MAb 2G11 and 3D9, instead of hybridoma culture fluids, was used as Control 1, and PBS instead of LCDV was used as Control 2. All reactions were performed in triplicate. The blocking rate was calculated as follows: $(OD_1 - OD_{Test})/(OD_1 - OD_2) \times 100$, where OD_1, OD_2 and OD_{Test} were the optical density (OD) values of Control 1, Control 2 and test treatments, respectively. Blocking rates ≥ 50% were considered to be positive results that suggested effective blocking of LCDV.

LCDV infection inhibition in FG cell culture

FG cells were grown in a 96-well plate at 22°C until the cell number reached about 10^4 well^{-1}. After the medium was removed, the cells were washed gently using MEM without FBS [MEM(–)] and incubated for 2 h at 22°C with increasing concentrations (0.01, 0.1, 1, 5 and 10 µg well^{-1}) or the same concentration (10 µg well^{-1}) of 100 µl anti-27.8 kDa protein MAbs. Following incubation, the cells were washed twice with MEM(–), LCDV at 4 $TCID_{50}$ or the increasing concentrations (8, 4, 2 $TCID_{50}$) in MEM(–) were added and incubated at 22°C for 1 h. After virus adsorption, the unattached viral particles were removed by washing with MEM(–), and 100 µl maintenance medium was added. The infected cultures were incubated for 5 d at 22°C and the cytopathic effect (CPE) in FG cells was monitored by phase contrast microscopy. MAb 1D5 against WSSV, instead of anti-27.8 kDa protein MAbs, served as Control 1, and MEM(–), instead of LCDV, served as Control 2. Each group was performed in 8 wells and the experiment was performed in triplicate.

Confocal fluorescence microscopy

FG cells in PBS were dropped on glass slides for 2 h at 22°C and then fixed in cold acetone for 10 min, followed by incubation with 40 µl positive hybridoma culture fluids for 60 min at 37°C in a moisture chamber. After washing 3 times with PBS, the cells were incubated for 60 min at 37°C in the dark with fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse Ig (Sigma) at a dilution ratio of 1:200 in PBS, containing Evan's blue dye (EBD, Fluka) as the counterstain. Slides were rinsed again, mounted with glycerin and observed under a laser scanning confocal microscope (LSCM, ZEISS LSM 510). MAb 1D5 against WSSV replaced positive hybridoma culture fluids as the negative control.

Immunogold electron microscopy (IEM)

FG cells were collected and fixed with 2% glutaraldehyde in PBS for 30 min. After washing with PBS, the cells were post-fixed with 1% osmium tetroxide, dehydrated with a graded ethanol series and embedded in Epon 812 (Imajoh et al. 2003). Ultrathin sections were picked up on nickel grids, washed with DW for 5 min, and incubated with 1% H_2O_2 for 10 min. Following 2 washes in DW and 3 washes in PBS, the sections were treated with a solution of 3% BSA in PBS for 1 h at 37°C. Subsequently, they were rinsed with PBST and incubated with positive hybridoma culture fluids or MAb 1D5 against WSSV as a negative control for 1 h at 37°C, and then washed with PBST again. The sections were incubated with goat-anti-mouse Ig conjugated with 10 nm colloid gold (Sigma) for 1 h at 37°C, stained with uranyl acetate for 30 min and lead citrate for 30 min after being washed with PBST and DW, and observed with a transmission electron microscope.

MAbs react with 27.8 kDa proteins in detected target tissues of LCDV

Flounder tissue samples, including gill, stomach, intestine, skin, kidney, spleen and liver, were cut into pieces about 1 mm^3, and added to ice-cold lysis buffer (0.02 M Tris-HCl, 0.137 M NaCl, 10% glycerol and 1% NP-40) with protease inhibitors (1 mM phenylmethyl sulfonyl fluoride, 2 mM EDTA, 10 mM NaF, 2 µg ml^{-1} Aprotinin and 5 µg ml^{-1} Leupetin). After ultrasonication treatment of 3 min, the homogenates were stirred on ice for 2h and centrifuged at 10,000 g for 20 min. Tissue proteins released into the supernatant were harvested, and the protein concentrations were quantified (Bradford 1976) and then subjected to SDS-PAGE in equal amounts of 10 µg $lane^{-1}$. After transferring to PVDF membrane and blocking with BSA, MAbs against 27.8 kDa protein were added as the primary antibody or MAb 1D5 against WSSV as the negative control, and AP-conjugated goat anti-mouse Ig served as the secondary antibody. The PVDF membrane was then stained with substrate solution containing NBT and BCIP. FG cell membrane proteins were used as positive controls.

RESULTS

Purification of 27.8 kDa protein

In the SDS-PAGE profile, about 30 FG cell membrane proteins were detected, with estimated molecular masses ranging mainly from 14 to 175 kDa according to marker (Fig. 1A, Lane 1). A protein with molecular mass of 27.8 kDa was purified (Fig. 1A, Lane 2) based on the result of co-immunoprecipitation with LCDV and FG cell membrane proteins (Wang et al. 2011). The protein concentration of purified 27.8 kDa protein was about 1 mg ml^{-1}.

Production of MAbs

After the fusion of myeloma and spleen cells, 162 wells yielded hybridomas, of which 13 hybridomas secreted 27.8 kDa protein specific monoclonal antibodies screened by ELISA. Then, 2 positive hybridomas designated as 2G11 and 3D9, which gave strong positive results (OD > 1.0), were cloned and subcloned by limiting dilution. All negative controls showed very low background levels (OD < 0.1). The concentrations of purified MAb 2G11 and 3D9 were 0.1mg ml^{-1}.

The results of western blotting showed that both MAb 2G11 and 3D9 reacted with only 1 band at a molecular weight of 27.8 kDa from all of the FG cell membrane proteins (Fig. 1B) and that the MAb 2G11 and 3D9 are all IgG type (data not shown).

Blocking effect of MAbs to LCDV

B-ELISA results showed that the blocking OD values of MAb 2G11 and 3D9 decreased and the blocking rate increased as the concentration of MAbs was increased (Table 1, Fig. 2). The OD value of Control 1 was about 0.55 in different concentrations of anti-WSSV MAb 1D5 and that of Control 2 was 0.0966. When incubated with the inhibition maximal concentration (10 µg $well^{-1}$) for each of the MAbs, the block-

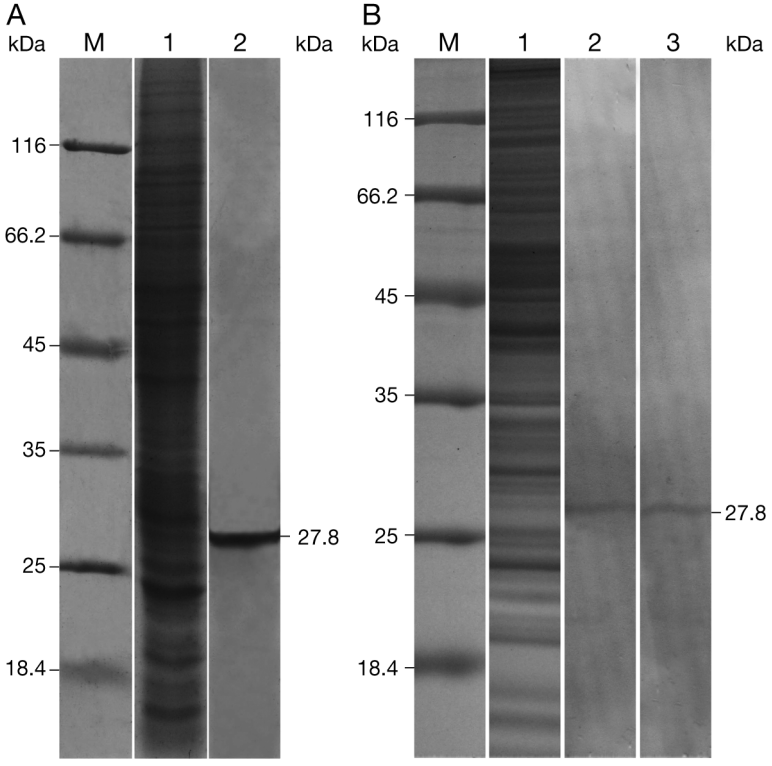

Fig. 1. *Paralichthys olivaceus.* (A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile of flounder gill (FG) cell membrane proteins (Lane 1) and purified 27.8 kDa protein (Lane 2). (B) Western blotting of FG cell membrane proteins with monoclonal antibodies (MAbs). Lane 1: Coomassie blue stained reduced SDS-PAGE of FG cell membrane proteins. Lanes 2, 3: Reaction with MAb 2G11 (Lane 2) and 3D9 (Lane 3) showed only one 27.8 kDa protein band. M: molecular mass marker

Table 1. *Paralichthys olivaceus.* Monoclonal antibodies (MAb) blocking effect at various concentrations, analyzed by blocking enzyme-linked immunosorbent assay (B-ELISA). Control 1: anti-white spot syndrome virus (WSSV) MAb 1D5; OD_{405}: optical density at 405 nm; BR: blocking rate. +: positive result, BR ≥ 50%; ±: equivocal result, BR = 30–49%; –: negative result, BR < 30%

MAb concentration	2G11		3D9		2G11 & 3D9		Control 1	Control 2
(μg well^{-1})	OD_{405}	BR (%)	OD_{405}	BR (%)	OD_{405}	BR (%)	OD_{405}	OD_{405}
0.01	0.4412	24.2 (–)	0.4933	12.7 (–)	0.457	20.7 (–)	0.5511	0.0966
0.1	0.3581	43.2 (±)	0.3815	38.1 (±)	0.3674	43.3 (±)	0.5567	0.0966
1	0.2409	68.2 (+)	0.2588	64.2 (+)	0.2508	70.4 (+)	0.5501	0.0966
5	0.1683	84.3 (+)	0.1735	83.1 (+)	0.1689	84.1 (+)	0.5531	0.0966
10	0.1655	84.6 (+)	0.1702	83.6 (+)	0.1483	88.4 (+)	0.5456	0.0966

ing rate (84.6 and 83.6%) was slightly lower than with that with co-incubation of the 2 MAbs (1:1) in 10 μg well^{-1} (88.4%) (Table 1), suggesting that the MAb 2G11 and 3D9 might recognize different epitopes on the 27.8 kDa receptor protein.

For the LCDV infection inhibition assay, firstly, FG cells were preincubated with the increasing concentration of MAb 2G11, 3D9 or a mixture of MAbs before adding 4 $TCID_{50}$ LCDV, and the MAbs were kept on cells throughout the infection assay to minimize viral spread. With the increasing concentration of anti-27.8 kDa MAbs, the levels of LCDV infection were reduced (Table 2A). On Day 5 post-infection (p.i.), in the presence of 5 or 10 μg well^{-1} MAb 2G11 and mixture MAbs (1:1), a few dying cells exhibited cell rounding and detachment (+, Fig. 3A); in the presence of 5 or 10 μg well^{-1} MAb 3D9 little CPE was present, which was characterized by cellular retraction, aggregation and loss of adherence to the substrate, followed by cellular disintegration (++, Fig. 3B). In contrast, in the presence of 5 or 10 μg well^{-1} MAb 1D5 against WSSV instead of MAbs against 27.8 kDa protein, CPE was clearly observed at Day 2 p.i. and then presented many large viral plaques at Day 5 p.i. (++++, Fig. 3D). No dying cells or CPE were observed in the uninfected control (Fig. 3E). Secondly, FG cells were preincubated with 10 μg well^{-1} MAbs and then infected with increasing concentrations of LCDV. The results of this showed the levels of LCDV infection increased with the increasing concentration of LCDV (Table 2B).

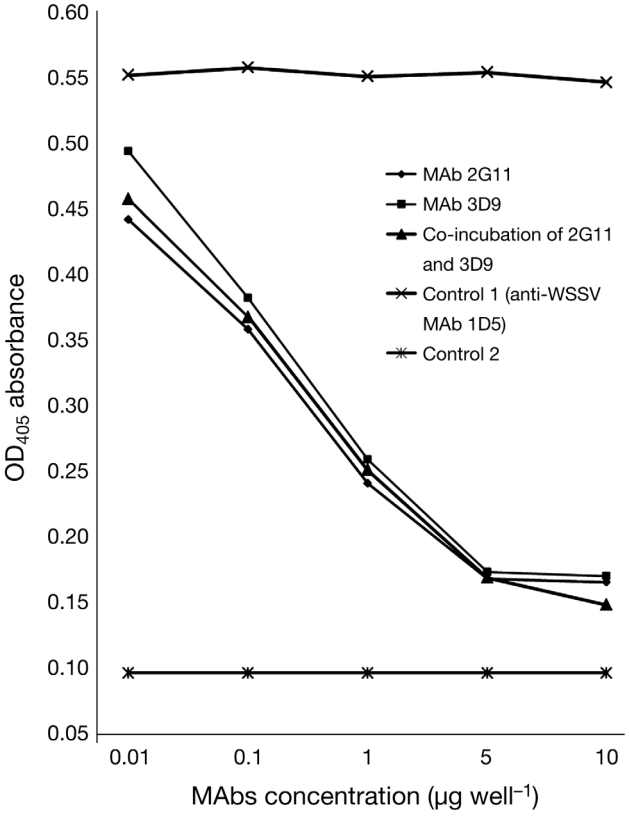

Fig. 2. *Paralichthys olivaceus.* Optical density at 405 nm (OD_{405}) absorbance of different blocking monoclonal antibody (MAb) concentrations by blocking enzyme-linked immunosorbent assay (B-ELISA). With the increasing concentration of MAbs, the blocking OD_{405} absorbance decreased. WSSV: white spot syndrome virus

Location of 27.8 kDa protein in FG cells

The immunofluorescence staining by using MAb 3D9 with FG cells showed that the specific green fluorescence signals were clustered at the membrane of FG cells (Fig. 4A), while staining using MAb 2G11 showed that the specific green fluorescence signals were present at the cell membrane uniformly (Fig. 4C). FG cells were stained red by EBD (Fig. 4B,D). No green fluorescence appeared in the negative control (Fig. 4E,F).

Transmission electron microscopy immunogold localization results showed that the high-density gold particles were mainly located on the membrane of the FG cells and occasionally in the cytoplasm near the cell membrane using MAb 3D9 (Fig. 5A,C,D).

Table 2. *Paralichthys olivaceus*. *In vitro* lymphocystis disease virus (LCDV) infection inhibition assays using anti-27.8 kDa protein monoclonal antibodies (MAbs). (A) Incubation with a constant concentration of LCDV and increasing concentrations of MAbs. (B) Incubation with a constant concentration of MAbs and increasing concentrations of LCDV. Control 1: anti-white spot syndrome virus (WSSV) MAb 1D5; ++++: 75–100% of cells showing cytopathic effect (CPE); +++: 50–75% of cells showing CPE; ++: 25–50% of cells showing CPE; +: few dying cells; –: no CPE

MAb type	MAb/LCDV concentration (μg well^{-1}/TCID$_{50}$)	CPE at days post injection 1	2	3	4	5
(A) LCDV conc. constant at 4 TCID$_{50}$; MAb conc. varied						
2G11	0.01	–	–	+	++	+++
	0.1	–	–	+	++	++
	1	–	–	–	+	++
	5	–	–	–	–	+
	10	–	–	–	–	+
3D9	0.01	–	+	+	++	+++
	0.1	–	–	+	++	+++
	1	–	–	–	+	++
	5	–	–	–	–	++
	10	–	–	–	–	++
2G11 & 3D9	0.01	–	–	+	++	+++
	0.1	–	–	+	+	++
	1	–	–	–	+	++
	5	–	–	–	–	+
	10	–	–	–	–	+
Control 1	0.01	–	+	++	++++	++++
	0.1	–	+	++	++++	++++
	1	–	+	++	++++	++++
	5	–	+	++	++++	++++
	10	–	+	++	++++	++++
(B) MAb conc. constant at 10 μg well^{-1}; LCDV conc. varied						
2G11	8	–	+	+	++	+++
	4	–	–	–	–	+
	2	–	–	–	–	–
3D9	8	–	+	+	++	+++
	4	–	–	–	–	++
	2	–	–	–	–	+
2G11 & 3D9	8	–	–	+	++	+++
	4	–	–	–	–	+
	2	–	–	–	–	–
Control 1	8	–	++	+++	++++	++++
	4	–	+	++	++++	++++
	2	–	–	+	+++	+++

Similar results were obtained by using MAb 2G11 (data not shown). In the control, no gold particles were observed (Fig. 5B,E).

27.8 kDa protein in detected target tissues of LCDV

In western blotting, the MAb 3D9 reacted with the 27.8 kDa protein in the tissues of gill, stomach, intestine and liver (Fig. 6A). Except for 3 other tissues (skin, kidney and spleen), as well as the negative control, no responses were observed (Fig. 6B). Similar results were also found by using MAb 2G11 (data not shown).

DISCUSSION

Previous work showed that polyclonal antiserum against 27.8 kDa protein was capable of inhibiting LCDV binding to FG cells, but it had cross-reactivity with the 42.7 kDa protein of FG cells (Wang et al. 2011). In this study, 2 MAbs (2G11 and 3D9) against 27.8 kDa protein of FG cells were produced, and western blotting demonstrated their specificity for the 27.8 kDa protein in the FG cell membrane proteins, which indicated that these MAbs would be suitable for virus infection inhibition assays.

Confocal fluorescence microscopy using MAb 2G11 and 3D9 revealed the presence of the 27.8 kDa protein on the membrane of FG cells. The 27.8 kDa protein was also confirmed on the membrane of the FG cells by IEM. These results provided evidence that the 27.8 kDa protein was mainly located at the surface of the FG cells. This indirectly suggests that the 27.8 kDa protein is the putative receptor specific for LCDV in flounder.

In the LCDV infection inhibition assay, the MAb 2G11, MAb 3D9, or a mixture of both could efficiently but not completely block the LCDV binding to FG cells, especially when the LCDV was at a higher concentration (8 TCID$_{50}$), the ability of anti-27.8 kDa protein MAbs to inhibit LCDV infection to FG cells was not significant compared with control MAb. These results suggested that the 27.8 kDa protein may be a putative receptor specific for LCDV infection of FG cells, but not necessarily the only one.

Gill, stomach, intestine, skin, kidney, spleen and liver are known to be the target tissues of LCDV in fish (Colorni & Diamant 1995, Kvitt et al. 2008, Zhan et al. 2010). In order to investigate the distribution of LCDV receptor-27.8 kDa in flounder, we extracted proteins from the target tissues of LCDV and performed western blotting. The 27.8 kDa protein was detected in gill, stomach, intestine and liver, but not in skin, kidney and spleen using MAbs against 27.8 kDa protein of FG cells as the probe. This may be because the skin, kidney and spleen have other cellular recep-

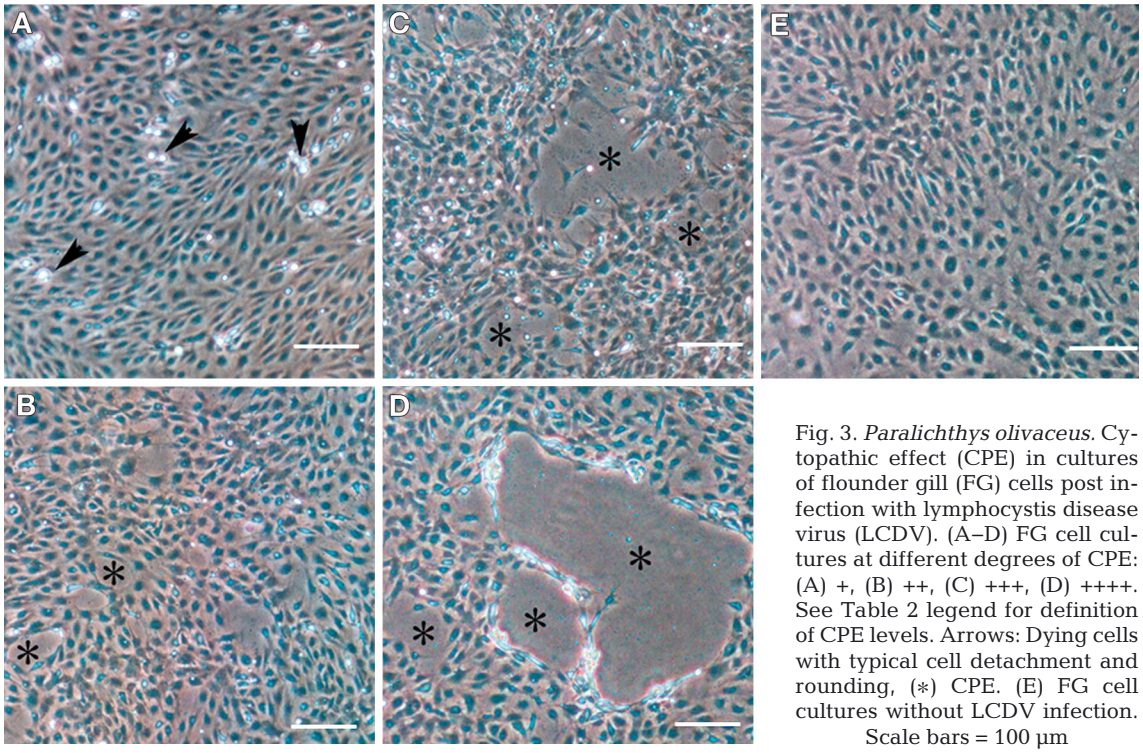

Fig. 3. *Paralichthys olivaceus.* Cytopathic effect (CPE) in cultures of flounder gill (FG) cells post infection with lymphocystis disease virus (LCDV). (A–D) FG cell cultures at different degrees of CPE: (A) +, (B) ++, (C) +++, (D) ++++. See Table 2 legend for definition of CPE levels. Arrows: Dying cells with typical cell detachment and rounding, (*) CPE. (E) FG cell cultures without LCDV infection. Scale bars = 100 µm

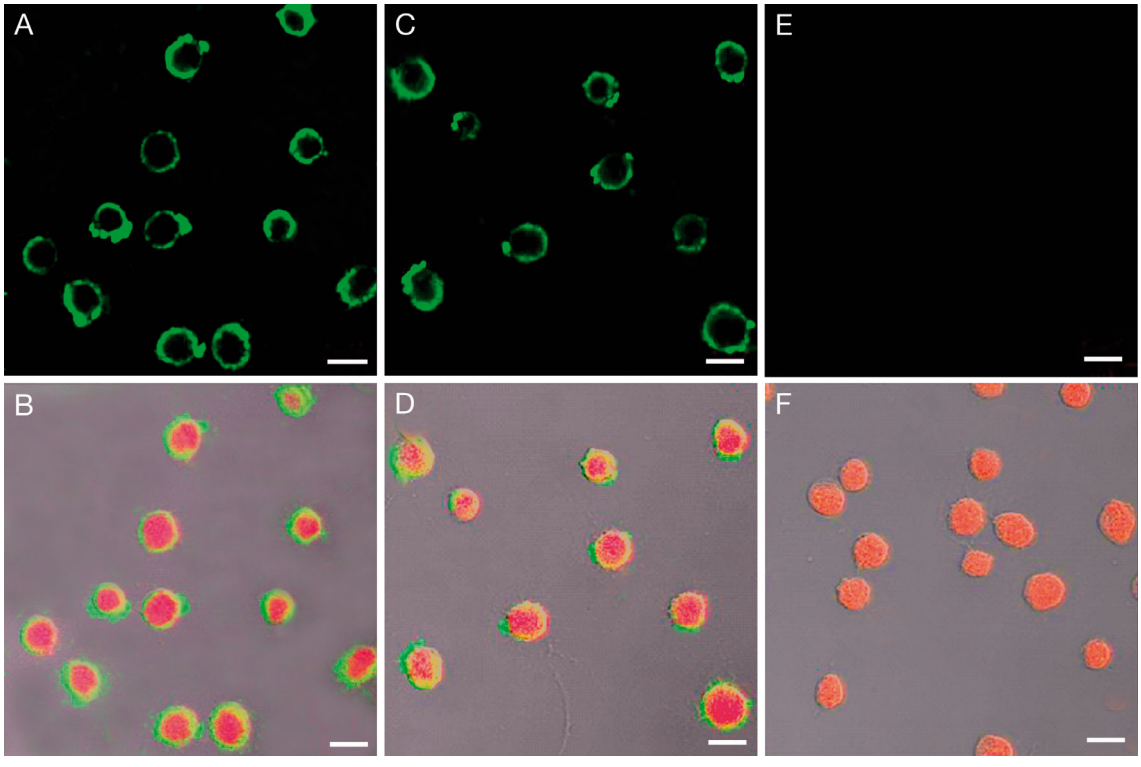

Fig. 4. *Paralichthys olivaceus.* (A–D) Confocal fluorescence microscopy and laser scanning confocal microscope views used to identify the 27.8 kDa protein recognized by monoclonal antibodies (MAbs) 3D9 and 2G11. The membranes of flounder gill (FG) cells showed green fluorescence, and all cells showed red fluorescence with (A,B) MAb 3D9 and (C,D) MAb 2G11. (E,F) When MAbs were replaced by MAb 1D5 against white spot syndrome virus (WSSV) as a negative control, all cells were (F) stained red with Evan's blue dye but (E) no green fluorescence was observed. Scale bars = 10 µm

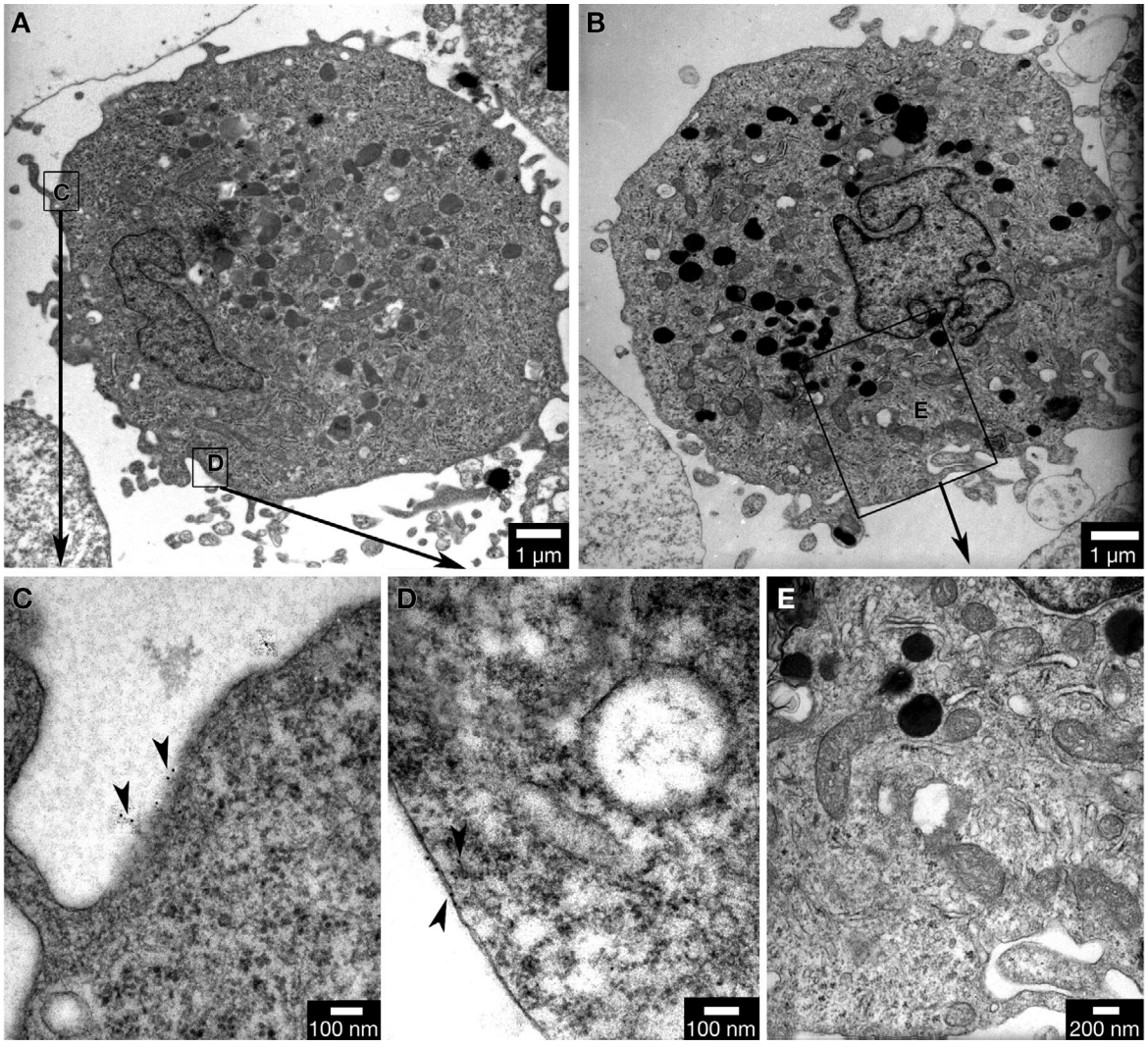

Fig. 5. *Paralichthys olivaceus.* Subcellular localization of 27.8 kDa protein in flounder gill (FG) cells by immunogold electron microscopy. (A) FG cell under transmission electron microscope using monoclonal antibody (MAb) 3D9. (B) No gold particles on the FG cell incubated with MAb 1D5 against white spot syndrome virus (WSSV). (C,D) Higher magnification view of insert area from (A). Arrows: 27.8 kDa protein immunolabeling, which was mainly observed on the plasma membrane and occasionally in the cytoplasm near the membrane. (E) Higher magnification view of insert area from (B)

tors to mediate LCDV infection, or the concentration of 27.8 kDa protein extracted from these tissues was too low to detect by western blotting, requiring other molecular methods for verification. Since lymphocystis nodules occur mainly on the skin and fins of flounder (Tidona & Daral 1999, Sheng & Zhan 2004), epidermal skin may contain other cellular receptors or need more sensitive methods to detect protein receptors, especially of infected tissue, so further research is needed.

In conclusion, we produced and characterized 2 MAbs (2G11 and 3D9) against the 27.8 kDa protein receptor of LCDV in FG cells. LCDV binding and infection were partly blocked by the MAbs and the 27.8 kDa protein was mainly located at the cell membrane, suggesting that the 27.8 kDa protein of FG cells played an important role in binding and infection of LCDV to FG cells. Moreover, several target tissues of LCDV in flounder confirmed the existence of a 27.8 kDa protein receptor of LCDV. We anticipate that these MAbs will prove useful in understanding virus–host cell interactions and blocking LCDV infection in fish.

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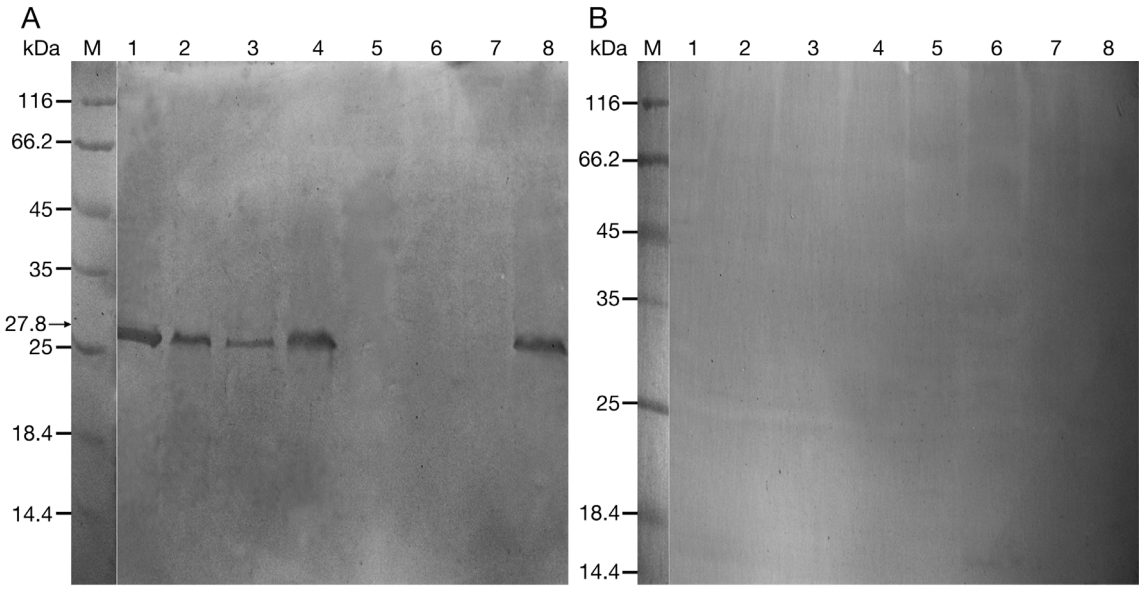

Fig. 6. *Paralichthys olivaceus.* (A) Monoclonal antibody (MAb) 3D9 and (B) the negative control, MAb 1D5 against white spot syndrome virus (WSSV), reacted with detected target organ proteins of lymphocystis disease virus (LCDV) in flounder by western blotting. Flounder gill cell proteins (Lane 1), gill (Lane 2), stomach (Lane 3), intestine (Lane 4) and liver (Lane 8) showed positive results with 27.8 kDa protein band and skin (Lane 5), kidney (Lane 6) and spleen (Lane 7) showed negative results. M: molecular mass marker

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