TEMPERATURE-DEPENDENT PATHOGENICITY OF GROUPER IRIDOVIRUS OF TAIWAN (TGIV)

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ABSTRACT

Grouper iridovirus of Taiwan (TGIV) is one of the most devastating infectious pathogens of grouper fry in Taiwan. As environmental temperature often plays an important role in the outbreak of diseases, we assayed the impact of different temperatures (18, 25 and 32°C) on TGIV infection both *in vitro* and *in vivo*. Cytopathic effect (CPE) characterized as cell rounding and lysis were observed in TGIV-infected grouper SB (swim bladder) cells at 25°C and 32°C, but was absent at 18°C. With temperature rising to 32°C, the presence of pycnotic nuclei and chromatin margination became prominent in the infected cells, indicating an apoptotic death. The morphological feature of apoptotic cell was further supported by the observation under electron microscope. In addition, the apoptosis of TGIV-infected cells promoted by hyperthermia (32°C) was confirmed by the assays of DNA laddering, DNA content and annexin V staining. To measure the temperature impact on the defense in TGIV-infected host, grouper fry was experimentally infected with the virus at 25°C and 32°C, respectively. Cumulative mortalities reached 100% in the fish held at 25°C on day 10 post-infection, whereas only 37.5% at 32°C at 2-week post-infection. In addition, the mortality in the infected fish of the 32-to-25°C group skyrocketed to 97.5% by day 8. All together, the data suggest different impacts of temperatures on the pathogenicity of TGIV in grouper: while the lower temperature (18°C) adversely affects the propagation of TGIV, the hyperthermic temperature (32°C) promotes apoptosis to prevent the spreading of virus, resulting in higher resistance of the host against the virus.

I. INTRODUCTION

Iridoviruses are DNA viruses that can infect various poikilothermic vertebrates (Chinchar, 2002). Piscine iridoviruses infect a wide range of fish and are classified into three genera: Lymphocystivirus, Ranavirus and Megalocytivirus (Wang et al., 2003). While lymphocystiviruses generally instigate non-fatal, superficial dermal infections, ranaviruses and megalocytiviruses are notoriously known for causing high mortality in many economic fish species, including red sea bream iridovirus (RSIV) of Japan, sleepy grouper disease (SGV) of Singapore, and infectious spleen and kidney necrosis virus (ISKNV) of China. In Taiwan, the incident of systemic infections of marine fishes caused by iridoviruses can be traced back to 1998. The infectious agents are isolated and designated as grouper iridovirus of Taiwan (TGIV) and grouper iridovirus (GIV), which belongs to the genus Megalocytivirus and genus Ranavirus, respectively (Chou et al., 1998; Chao et al., 2002; Murali et al., 2002). These two agents are proved to be of high virulence to different teleost fishes in aquaculture. Interestingly, the natural outbreak occurs most frequently during spring and summer (Wang et al., 2003; Li et al., 2011).

Temperature-associated outbreaks have been reported in several piscine iridoviruses. For the ISKNV in China, the outbreaks have occured only from March to November in 1994-1998, since its first incidence in 1994 (He et al., 1998; He et al., 2000). ISKNV is one of the most important etiological agents in the mandarin fish (*Siniperca chuatsi*) industry in China. He and collogues discovered that ISKNV causes no clinical signs or mortality in mandarin fish held at 20°C and 15°C. However, when the water temperature was risen to 25°C, significant mortality was observed (He et al., 2002). The observation is consistent with the natural outbreaks of ISKNV in the field. In addition, similar observation was reported in epizootic haematopoietic necrosis virus (ENHV) (*Epinephelus malabaricus*) infection. ENHV is the first virus isolated from fish in Australia. It is a member of Ranavirus that causes severe systemic disease in feral and cultured fish species, such as red fin perch (*Perca uviatilis*) and rainbow trout (*Oncorhynchus mykiss*) (Langdon et al., 1986; Langdon et al., 1988). Viral transmission experiments showed that the incubation period of EHNV in redfin perch was 11 days at a water temperature of 19-21°C,

while longer (up to 28 days) at colder temperatures. No occurrence of disease was observed when the temperature was below 12°C (Whittington and Reddacliff, 1995).

In this study, we demonstrated that apoptosis occurred in TGIV-infected grouper cells in a temperature-dependent manner. Analyses by electron microscopy, DNA laddering, DNA content, and annexin-V florescent staining showed that apoptosis prevailed at 32°C, but not at 25 and 18°C. Most interestingly, we found that fish was more resilient to the virus when held at 32°C, indicating that apoptosis might serve as an advantageous factor to the host defense against TGIV.

II. MATERIALS AND METHODS

1. Cell Culture and Virus

Grouper iridovirus of Taiwan (TGIV) was isolated from diseased grouper (*Epinephelus sp.*) in southern Taiwan (Chou et al., 1998). A TGIV-susceptible cell line swim bladder (SB) established from the swim bladder of Malabar grouper (*Epinephelus. malabaricus*) was used for virus multiplication and infectivity assays. SB cells were cultured in Leibovitz's L-15 medium supplemented with antibiotics (penicillin 50 I.U./ml, streptomycin 50 μg/ml, fungizone 1.75 μg /ml) at 25°C. For routine passage, the medium was supplemented with 10% fetal bovine serum (FBS), and for virus propagation and titration, 2%. The infectivity assay was conducted with titration technique and the titer was expressed as $TCID_{50}$/ml (50% tissue culture infective dose) (Roveozzo and Burke, 1973).

2. Morphological Observation of TGIV-Induced CPE at Different Temperature

TGIV was inoculated into SB cells at multiplicity of infection (MOI) of 1 and held at 18°C, 25°C, 32°C, respectively. Cytopathic effect (CPE) was observed daily and on day 5 post-infection, infected cells from 3 incubation temperatures were observed under an Olympus IX71 inverted microscope and photography.

3. Electron Microscopic Observations of TGIV-Infected Cell Cultures at Different Temperature

Infected SB cells (MOI of 1) maintained at 18°C, 25°C, 32°C were collected on 5 days post-infection (dpi) and were immersed in cacodylate buffered 5% glutaraldehyde for 1.5 h at 4°C for primary fixation. Samples were then washed three times with cacodylate buffer, and placed in 1.0% osmium tetroxide for secondary fixation. Samples were subsequently dehydrated in serial concentrations of ethanol, and the dehydrated samples were embedded in Spurr resin. Ultra-thin sections were cut from the embedded samples, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (H-7100, Hitachi).

4. Detection of Apoptotic Cells

1) DNA Laddering

SB cells were seeded into 25 cm² plastic tissue-culture flasks and incubated at 25°C overnight. After TGIV inoculation (MOI of 1), cells were maintained at 18°C, 25°C, 32°C and sampled on 1, 3, 5, 7, 9 dpi. Infected cells were harvested by centrifugation at 4°C at 800 ×g for 5 min. The cell pellets were lysed with NP-40 lysis buffer (50 mM Tris-HCl, 20 mM EDTA and 1% NP-40, AMRESCO). SDS (sodium dodecyl sulfate, final concentration is 1%, Nihon Shiyaku and Worthington) and proteinase K (final concentration is 2.5 mg/ml, Worthington) were added to the lysates and the mixture was incubated at 37°C for 3 h. DNA was precipitated by adding 99.5% ethanol (final concentration is 70%, Nihon Shiyaku) and stored overnight at -80°C. The precipitated DNA was centrifuged at 16,000 ×g for 30 min at 4°C, and electrophoretically separated on a 2% agarose gel.

2) Loss of DNA Content

Because TGIV-infected cells incubated at 18°C did not show apoptotic features, the assay to measure loss of DNA content from apoptotic cells was carried out only in infected cells (MOI of 1) maintained at 25°C and 32°C. At 1, 3, 5, 7, 9 dpi, cell were washed twice with phosphate buffered saline (PBS), harvested by trypsin, suspended, and fixed in 80% ethanol at -20°C for 24 h. The fixed cells were thawed quickly at room temperature and collected by centrifugation (800 ×g, 4°C, 5 min). The cells were then washed twice with PBS and resuspended in extraction buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) for 5 min, followed by centrifugation (800 ×g, 4°C, 5 min). The pellets were resuspended in PBS containing RNase A (100 μg/ml) and propidium iodide (PI) (50 μg/ml) for 30 min, and the DNA content of apoptotic cells were determined by flow cytometer (BD FACSCanto). The data were then expressed as means from three independent experiments and analyzed by T-test.

3) Identification of Necrosis and Apoptosis by Annexin V Staining

SB cells were seeded onto aseptic round coverslips placed in 24-well plate and incubated at 25°C overnight. After TGIV inoculation (MOI of 1), cells were maintained at 18°C, 25°C, 32°C and sampled at the indicated time points post-infection by removing the coverslips out from the plate. The coverslips were rinsed gently with PBS and stained with PBS containing Annexin V-FITC and PI (10 mol/L) in the dark at room temperature for 30 min. The cells were then observed under a fluorescence microscope (Olympus) [excitation 488 nm, emission > 515 nm for Annexin V-FITC (green) and PI (red)]. In addition, the percentage of apoptotic cells was measured by flow cytometry (BD FACSCanto).

5. Effect of Temperature on TGIV Infection *in vivo*

Grouper fry averaged 3.31 cm in body length and 0.60 g in body weight were reared in 30 L glass tanks (10 individuals per tank). Each glass tank contained filtered and aerated seawater with a salinity of 25-30 part per thousand (ppt), and was
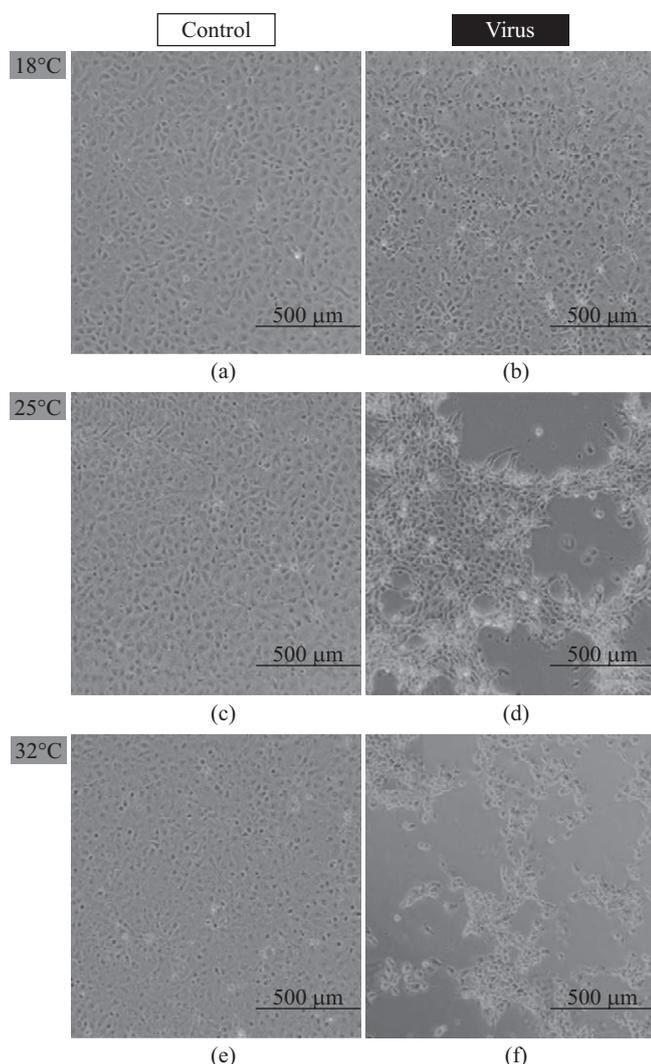
Fig. 1. TGIV-induced cytopathic effect (CPE) in grouper SB cells at 18°C ((a) and (b)), 25°C ((c) and (d)) and 32°C ((e) and (f)). The cell morphology of mock-infected ((a), (c) and (e)) and TGIV-infected ((b), (d) and (f)) cells were observed under inverted microscope. The CPE characterized as cell rounding and lysis was observed in TGIV-infected cells at 25°C and 32°C, but was absent at 18°C.
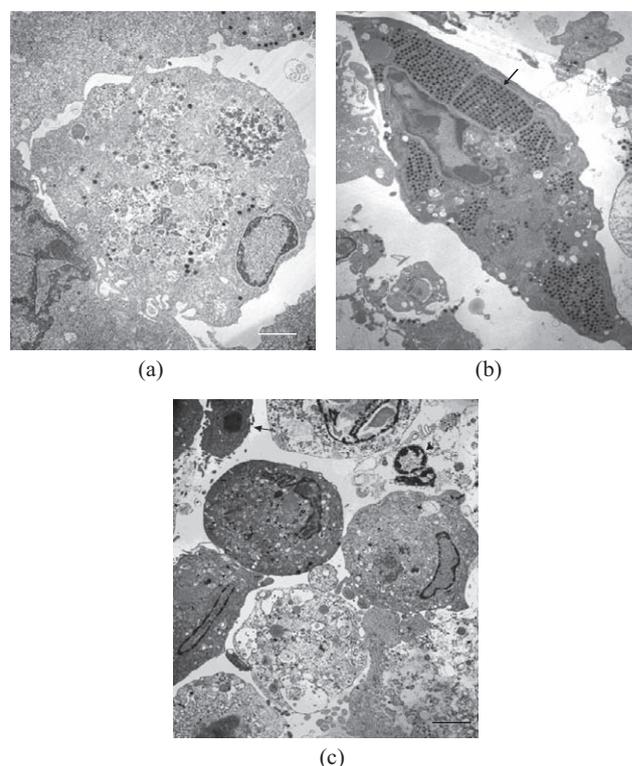

Fig. 2. Electron micrographs of TGIV-infected SB cells cultured at 18°C, 25°C and 32°C CPE cells. At 18°C, heterochromatin clustering under nuclear envelope (indicated by white arrowhead) and few virus particles (indicated by arrow) in the cytoplasm were found in the infected cell (a). At 25°C, significant heterochromatin clustering under nuclear envelope (indicated by white arrowhead) and a large quantity of viruses in paracrystalline arrays (indicated by arrow) were found in the cytoplasm of infected cell (b). At 32°C, nuclear chromatin condensation (indicated by arrow) and apoptotic bodies (indicated by arrowhead) were evident (c). Bar = 1 μm.

III. RESULT

1. Cytopathic Effect Caused by TGIV in Grouper Cells at Different Temperatures

The morphological changes associated with TGIV infection in grouper cells were first assayed under optical light microscope. As shown in Fig. 1, CPE characterized as cell rounding and lysis was observed in the TGIV-infected SB cells cultured at 25 and 32°C, but not in cells at 18°C. The development of CPE appeared to be faster in the infected cells at 32°C than 25°C. In fact, complete lysis was observed in the infected cells cultured at 32°C by day 5 post-infection.

The morphological change in the TGIV-infected cell was investigated further by transmission electron microscopy. At 5 dpi, heterochromatin clustering at the nuclear envelope was found in both the infected cells cultured at 18 and 25°C (Figs. 2(a) and (b)). The propagation of virus was evident as demonstrated by the scattering virions in the cytoplasm of infected cells kept at 18°C. At 25°C, abundant scattered virions were found clustering in paracrystalline arrays, which likely represent viral maturation sites. (Figs. 2(a) and (b)). Contrary to

placed in water bath for temperature control. The grouper fry were fed twice a day. Three replicates of twenty of grouper fry were injected intraperitoneally with $1 \times 10^{3.5}/0.1$ ml TCID$_{50}$ of TGIV. The fish injected with L-15 medium (containing 2% FBS) served as control. After challenge, fish were reared at 25 ± 0.5°C and 32 ± 0.5°C, respectively. In addition, another group of fry was maintained at 32°C after infection for 12 hrs and then transferred to 25°C. Water salinity was 25-30 ppt throughout the experiment and the mortality was observed daily. Data was analyzed by SPSS (version 22) statistical software package for Windows. Duncan's multiple range test was employed to determine the significance of difference with in treatments. For each treatment, 3 determinations were used and the mean values were calculated. Values were considered to be significantly different at $p < 0.01$ (Norušis and SPSS Inc., 1990).
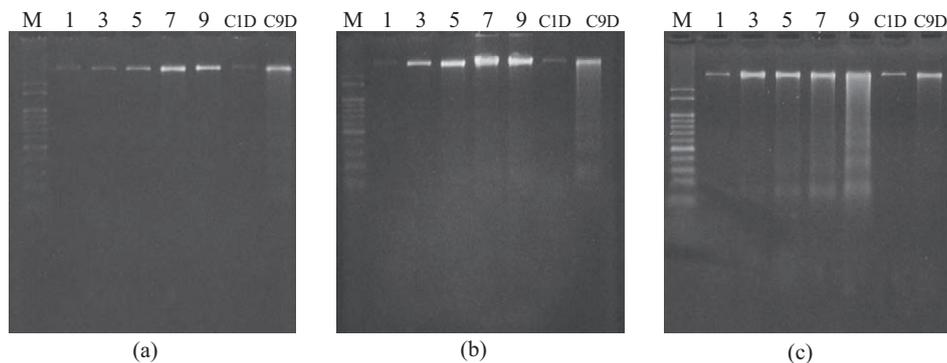
(a)   (b)   (c)

Fig. 3. DNA laddering in TGIV-infected SB cells at 18°C (a), 25°C (b) and 32°C (c). DNA was extracted from TGIV-infected SB cells and electro-phoresed on 2% agarose. M: molecular weight marker. The number above each lane indicates the day when DNA was extracted from infected cells after infection (1, 3, 5, 7. 9 dpi). C1D and C9D indicate the DNA extracted from control cells on day 1 and day 9, respectively. Fragmented DNA was evident only in the infected cells cultured at 32°C.

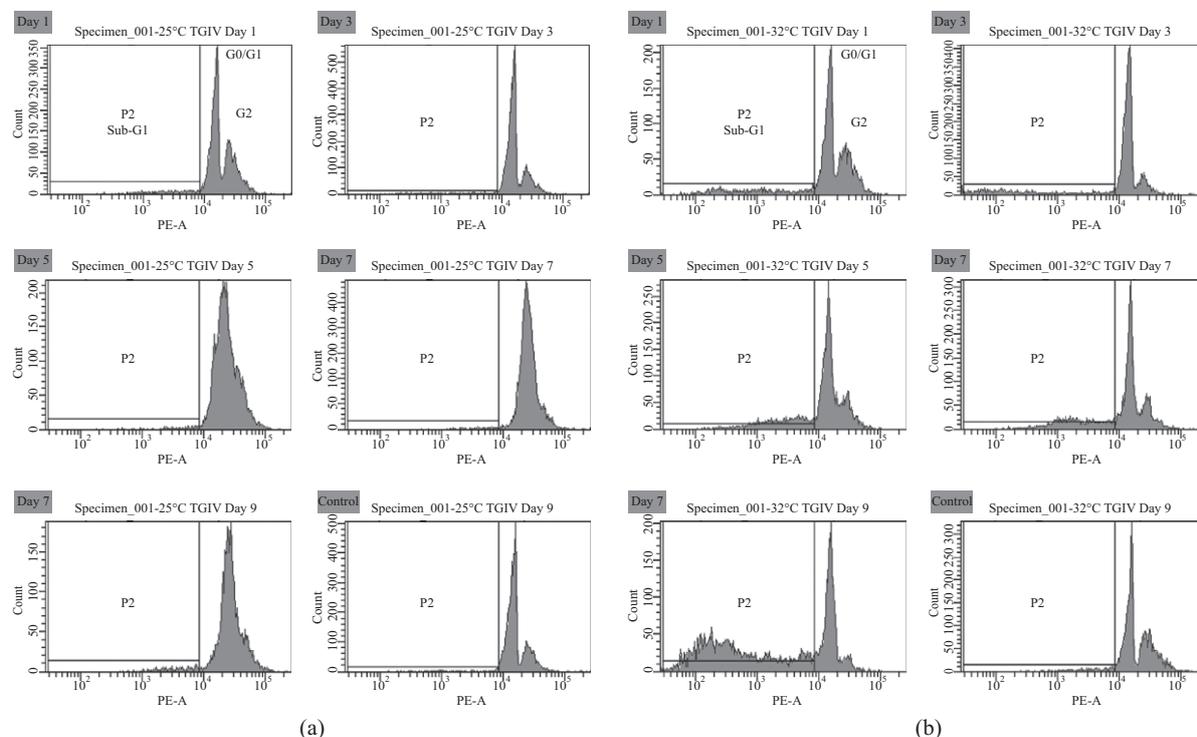(a)   (b)

Fig. 4. DNA content of TGIV-infected cells cultured at 25°C (a) and 32°C (b). TGIV-infected cells were harvested, fixed and stained with PI on day 1, 3, 5, 7 and 9 post-infection. Mock-infected cells from 9 dpi were included for comparison. The cellular DNA content was measured by flow cytometry. Due to the change in DNA content caused by fragmentation, the apoptotic cell can be identified as the hypoploid sub- G0/G1 peak locating to the left of the normal diploid G0/G1 peak (P2).

the observation found in the cells cultured at 18 and 25°C, less amount of assembled virions presented in the infected cells incubated at 32°C; instead, chromatin condensation and apoptotic body-like vesicles were found in these cells (Fig. 2 (c)). These observations indicate that higher temperature would promote apoptosis in infected grouper cells.

2. Verification of TGIV-Induced Apoptosis in Grouper Cells at High Temperature

The mode of cell death was investigated further using DNA laddering, DNA content, and Annexin V-PI staining assays. For DNA laddering, genomic DNA was isolated from TGIV-infected and control cells cultured at different temperatures, separated by electrophoresis, and visualized with ethidium bromide. As shown in Fig. 3, DNA laddering was not observed in the infected cells incubated at 18°C and 25°C at 1, 3, 5, 7 and 9 dpi. Fragmented DNA was not detected in the control cells at all three temperatures as well. On the other hand, DNA laddering was found in the infected cells incubated at 32°C at as early as 3 dpi.
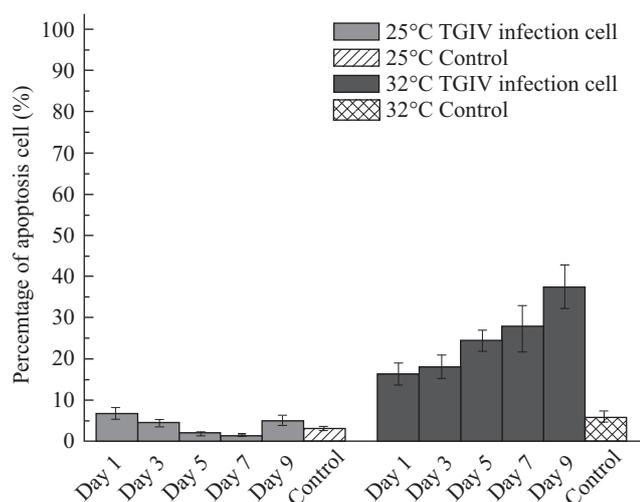
Fig. 5. Comparison of the temperature effect on the prevalence of apoptosis in TGIV-infected grouper SB cells. The quantity of the sub-G0/G1 peak was measured and plotted. The percentage of apoptotic cells in the infected cells at 32°C steadily increased as the infection progressed, reaching 37.5% by day 9 post-infection. On the other hand, the percentage of apoptotic cells at 25°C remained less than 10% throughout the period of time. (*, p < 0.05-0.01; **, p < 0.01).

Another indicator of apoptotic cell is the loss of DNA content caused by DNA fragmentation, which can be readily measured by flow cytometry. The apoptotic cell can be identified as the hypoploid sub-G0/G1 peak locating to the left of the normal diploid G0/G1 peak. As shown in Fig. 4, the population of apoptotic cells increased as the temperature shifted from 25 to 32°C. The percentage of apoptotic cells in the infected cells at 32°C steadily increased as the infection progressed, reaching 37.5% by 9 dpi (Fig. 4(b) and Fig. 5). On the other hand, the percentage of apoptotic cells at 25°C remained less than 10% throughout the period of time (Fig. 4(a) and Fig. 5).

During the early stage of apoptosis, the phosphatidylserine (PS) from the inner leaflet of plasma membrane is translocated to the outer leaflet where the PS can readily be stained with annexin V. When the cell progresses into late apoptotic stage, the membrane becomes permeable to PI, allowing the staining of cell with both annexin V and PI. Generally, cell stained with negative annexin V and positive PI is regarded as necrotic cell. As shown in Fig. 6, the population of annexin V+/PI- cells remained steady in the TGIV-infected cells cultured at 25°C, while the population at 32°C gradually increased to 24% on 9 dpi when the population in the uninfected cells was 5.6%. Interestingly, at 25°C the population of annexin V-/PI+ cells steadily increased in the infected cells, reaching 25% on 9 dpi, whereas only 2% in the uninfected cells. These data indicate that TGIV induces necrosis in grouper SB cells at 25°C but promotes another mode of death, i.e. apoptosis, in the cells when the water temperature is shifted to 32°C.

3. Temperature Effect on TGIV Enfection *in vivo*

The impact of temperatures on the resistance to TGIV was investigated further *in vivo* by experimental infection of the fish with the virus at 25°C and 32°C, respectively. The third group of fish was infected at 32°C but was transferred to 25°C 12 hrs later. As shown in Fig. 7, the average cumulative mortality in the infected fish at 25°C reached 100% by 10 dpi. Surprisingly, while the average mortality in the infected fish at 32°C was 37.5%, the mortality in the infected fish of the 32-to-25°C group skyrocketed to 97.5% by day 8. Statistical analysis further showed that the mortality in the 32°C group was significantly lower than that in the 25°C and 32-to-25°C groups. The data clearly demonstrated the dramatic effect of temperature on the pathogenicity of TGIV.

IV. DISCUSSION

Temperature is often a critical environmental factor to the outbreak of viral diseases. In this study, we demonstrated that ambient temperatures exert different effects on the pathogenicity of TGIV both *in vitro* and *in vivo*. We showed that the CPE induced by TGIV in grouper SB cells was temperature-dependent (Fig. 1), as demonstrated by that the progression of CPE in the infected cells was faster at 32°C than 25°C, while absent at 18°C. Examination by electron microscopy further revealed ultrastructural morphological features typical to apoptosis in the infected cells at 32°C (Fig. 2), indicating that TGIV might prompt apoptosis in the infected cells under high temperature. The apoptotic death was subsequently confirmed by analyses of DNA laddering, DNA content and annexin V-PI staining. Intriguingly, necrosis instead of apoptosis was predominant in the TGIV-infected cells cultured at 25°C. To our best knowledge, this is the first demonstration of temperature-dependent mode of death caused by aquatic iridoviruses. Nonetheless, we noticed slight sign of DNA laddering in the control samples (C9D) in Figs. 3(a) and 3(b). We cannot assert the cause of the DNA ladders found in the control cells. It was not likely caused by contamination of virus as we did not find evidence of CPE in the C9D samples (data not shown). One potential explanation is that the apoptosis was caused by the undernourishment due to long incubation time with low content of FBS. The phenomenon has been shown in other cell lines, such as rat proximal tubule (RPT) cells. Li and colleagues showed that the decrease in FBS content from 10% to 0.2% could induce 14% of the RPT cells undergoing apoptosis (Li et al., 2006).

Apoptosis is a genetically controlled process of cell self-destruction. It generally occurs during embryogenesis, tissue forming, immune regulation and many other physiological processes (Wyllie et al., 1980; Duvall and Wyllie, 1986). Pathological stimuli, such as radiation damage, oxidative stress and viral infection can also induce apoptosis (Inoue et al., 1997; Liu et al., 2003; Chu et al., 2004). The interaction between viruses and host is tremendously complex in its nature. Apoptosis is a double-edged sword to both virus and host in their interaction. Apoptosis might be beneficial to
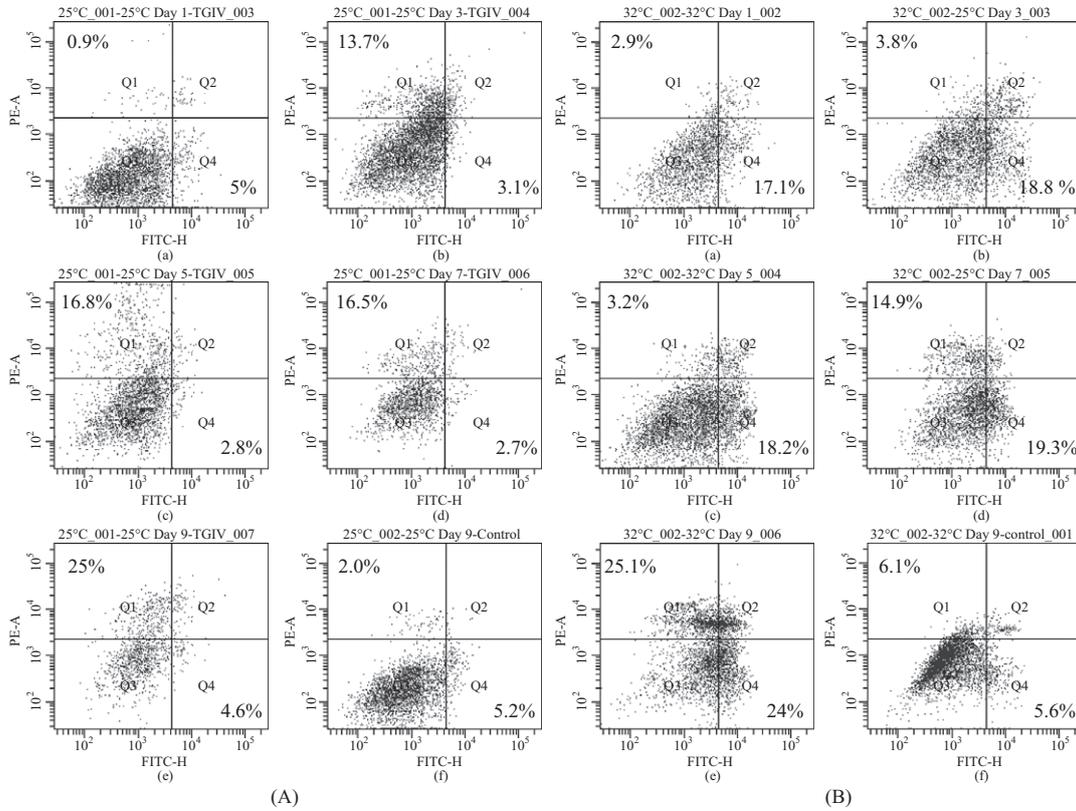
Fig. 6. Annexin V-PI staining of TGIV-infected SB cells cultured at 25°C (a) and 32°C (b). For each graph, the y-axis represents percentage of cells stained by PI, which stains the necrotic cells, and the x-axis represents percentage of cells stained by Annexin V-FITC, which stains the early apoptotic cells. Cells that are only stained with PI (upper-left quadrant) are necrotic. Cells stained with only Annexin V-FITC (lower-right) are in early apoptosis. Cell stained with both PI and Annexin V-FITC (upper-right) cannot be differentiated between necrotic or late apoptosis. Cells that do not stain with either PI or Annexin V-FITC are viable (lower-left). The population of annexin V+/PI- cells remained steady in the TGIV-infected cells cultured at 25°C, while the population of annexin V-/PI+ cells steadily increased. The population of annexin V+/PI- cells gradually increased in the TGIV-infected cells cultured at 32°C.
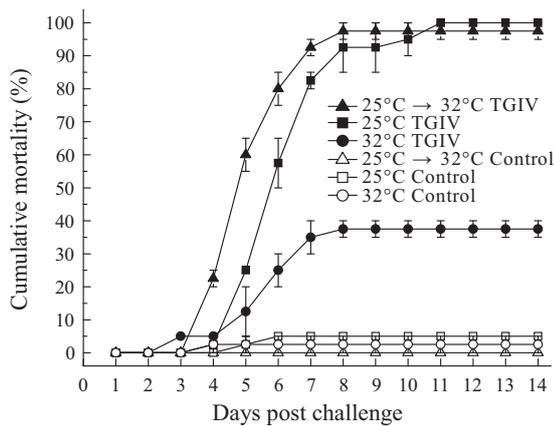

Fig. 7. Cumulative mortalities in TGIV-infected grouper reared at different temperatures. The impact of temperatures on the pathogenicity of TGIV was investigated in vivo by experimental infection of grouper fry with the virus at 25°C and 32°C, respectively. Another group of virus-infected fish was maintained at 32°C for 12 hrs and transferred to 25°C. For viral challenge, twenty fish were injected intraperitoneally with TGIV at the dose of $1.0 \times 10^{3.5}$ TCID$_{50}$/0.1ml/fish or L15-2 medium. Data shown are the mean of three replicates, a-c: values with different letters are differ significantly (p < 0.01).

the host by means of eliminating virally infected cells (Chelbi-Alix et al., 1998). On the other hand, virus might evade the host immune system by triggering apoptotic death of immune cells (Barber, 2001). Many viruses have evolved mechanisms to suppress the apoptosis in infected cells to sustain their own propagation (Shen and Shenk, 1995; Teodoro and Branton, 1997). It has been shown that the B-cell lymphoma (Bcl)-2-like gene encoded by GIV genome can suppress the apoptosis in grouper cells after infection (Lin et al., 2008; Pham et al., 2012). It is yet to be verified if such anti-apoptotic gene(s) is also encoded by the TGIV genome.

Temperature can affect viral pathogenesis in different ways, for examples, virus replication, infectivity pattern and transmission, as well as host immune response (Harrington et al., 1994; White, 1994; Eick and Hermeking, 1996; Oldstone, 1997). Our study showed that while the TGIV-induced CPE progressed much faster in cells cultured at 32°C than 25°C, the amount of assembled virions was far less in cells at 32°C as compared to 25°C (Fig. 2). This temperature effect is different from that associated with nervous necrosis virus (NNV), a betanodavirus that also causes tremendous mortality in grouper. Chi and colleagues have demonstrated that higher temperature

suppresses both the development of CPE and the yield of viruses in the NNV-infected grouper GF-1 cells (Chi et al., 1999). On the other hand, Granja and colleagues investigated the mean apoptotic index (AI) in White Spot Syndrome Virus (WSSV) infected white shrimp (*Litopenaeus vannamei*) maintained at 25°C or 32°C (Granja et al., 2003). The data indicated that hyperthermia might facilitate the occurrence of apoptosis in the WSSV-infected shrimp cells. Of note, both grouper and white shrimp are tropical species. The different outcomes caused by temperatures might be attributed to the different nature of viruses and host cells. Nevertheless, it is clear from our study that the faster progression of apoptosis induced by high temperature in the TGIV-infected cells restricted the propagation of the virus.

It is likely that higher temperature might be beneficial to the host to combat against TGIV infection. Indeed, the data of our challenge experiment showed that while TGIV led to higher cell death rate in vitro at 32°C than 25°C, the survival rate of infected grouper fry was actually much higher at 32°C. Furthermore, when the fish was transferred from 32°C to 25°C after infection the mortality drastically increased to almost as high as that in the fish maintained at 25°C. It is thus tempting to speculate that apoptosis might be employed by the host to suppress the spreading of TGIV in the body to promote the survival rate of host. In many viral diseases, apoptosis plays an important role in the resistance of host against the infection, but the outcome varies depending on the nature of virus and host. Similar to the case of TGIV, the survival rate is higher in the WSSV-infected white shrimp cultured at 32°C than other lower ambient temperatures (Ponce-Palafox et al., 1997). Furthermore, the increased survival rate in WSSV-infected shrimp at high temperature coincides with the reduction in viral yield in the infected individuals (Granja et al., 2003; Granja et al., 2006).

In summary, we present here the first report on the temperature-dependent pathogenicity of TGIV in grouper. At the optimal temperature (25°C), TGIV induces necrosis in the infected grouper cells; however, the virus promotes another mode of death, apoptosis, when the temperature rises to 32°C. On the other hand, grouper fry becomes more resilient to the infection at 32°C, indicating that apoptosis might serve as an advantageous factor to the host defense against TGIV by eliminating virally infected cells. Our future study will focus on the molecular mechanism underlying the temperature-dependent pathogenicity of TGIV.

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