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Perforin-dependent cytotoxic mechanism in killing by CD8 positive T cells in ginbuna crucian carp, *Carassius auratus langsdorfii*

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ABSTRACT

T cell-mediated cytotoxicity occurs via pathways based on perforin or Fas mechanisms. Perforin is a protein present in the cytoplasmic granules of $CD8^+$ cytotoxic T lymphocytes and is secreted to form pores on target cell membranes. In fish, although the involvement of perforin in cytotoxicity have been suggested for several species, perforin-mediated cytotoxicity of $CD8\alpha^+$ lymphocyte in conjunction with expression of the perforin gene has not been reported. In order to investigate the killing mechanism of $CD8\alpha^+$ lymphocytes by perforin-mediated pathway in fish, we measured apoptosis of target cells triggered by $CD8\alpha^+$ lymphocytes, performed cytotoxic assays in the presence or absence of perforin inhibitor; concanamycin A and EGTA, and analysed the expression of *perforin1*, *perforin2* and *perforin3* isotypic genes in ginbuna crucian carp. In the present study, we found that CTLs attached with target cells. CTL should have direct contact with target cells to kill them. Approximately 50% of target cells were positive for annexin V after co-cultured with $CD8\alpha^+$ lymphocytes, indicating the induction of apoptotic cell death. Concanamycin A, which induces depolymerization of perforin resulting in lytic function, suppressed the cytotoxicity of $CD8\alpha^+$ cells in a dose-dependent manner. In addition, cytotoxicity mediated by $CD8\alpha^+$ lymphocytes were significantly suppressed by the addition of the Ca^{2+}-chelating agents EGTA or EGTA–Mg^{2+}, and the addition of Ca^{2+} restored the killing mechanism of target cells. We further found enhanced expression of *perforin1* but not *perforin2* or *perforin3* in CTLs from allo-sensitized fish. The present study has demonstrated that ginbuna CTLs kill target cells through perforin-mediated pathway, suggesting that perforin-mediated pathway is conserved throughout vertebrate.

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1. Introduction

Cytotoxic T lymphocytes (CTLs) are effector lymphocytes that play invaluable roles in defence against virus-infected or transformed cells. CTLs kill their cellular targets by either of two mechanisms that both require direct contact between the effector and target cells, i.e. the secretory and non-secretory pathways. The latter pathway involves the engagement and aggregation of target cell death receptors such as Fas by their cognate ligands, (e.g. Fas ligand, FasL) on the killer-cell membrane, which results in classical caspase-dependent apoptosis (Nagata and Golstein, 1995). In the secretory pathway, cytoplasmic granular toxins, predominantly a calcium-dependent membrane-disrupting protein known as perforin, and a family of structurally related serine proteases (granzymes) are secreted by exocytosis and together induce apoptosis of the target cell (Ishiura et al., 1990; Trapani and Smyth, 2002). A positive correlation exists between perforin/granzyme expression and activated mammalian CTL (Cuesta et al., 2003; Garcia-Sanz et al., 1988; Kelso et al., 2002; Liu et al., 1990; Lowrey et al., 1989).

A perforin-like molecule has been discovered in Japanese flounder (Hwang et al., 2004), trout (Athanasopoulou et al., 2009) and ginbuna (unpublished), and perforin gene expression has been identified in their lymphoid tissues. In Japanese flounder, lytic activity of recombinant perforin protein has been detected in the presence of calcium (Hwang et al., 2004). Furthermore, earlier studies have indicated that calcium plays an important role in the cell-mediated cytotoxic response in catfish (Zhou et al., 2001) and carp (Companjen et al., 2006), and inhibitors of perforin or Fas of the cytotoxic pathway suppressed cytotoxic activity of T cell clones (Zhou et al., 2001). These studies strongly suggest that pathways of killing similar to those of mammals are operative in fish.

It has been reported that T cell-enriched leukocytes (surface IgM (sIgM) negative cells) are able to execute specific cell-mediated cytotoxicity (CMC) against virus-infected cells in ginbuna (Somamoto et al., 2006) and against allogeneic cells in rainbow

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trout (Fischer et al., 2003). The presence of alloantigen-specific cytotoxic T cell clones and cytotoxic mechanisms have also been reported in channel catfish (Zhou et al., 2001). Furthermore, it has been demonstrated that cells expressing CD8α and TCRβ mRNA kill allogeneic cells (Fischer et al., 2003) and virus-infected cells (Somamoto et al., 2006, 2009). In ginbuna, the expression of these genes is enhanced by sensitization with alloantigen or virus infection. Recently, we demonstrated that the specific CMC of ginbuna leukocytes against allogeneic target cells is executed only by CD8α$^+$ cells (Toda et al., 2009). Although cytotoxicity of teleost CD8$^+$ cells similar to that of mammalian CTL has been demonstrated, their killing mechanisms remain unknown. Despite previous research suggesting the possibility of a perforin-dependent cytotoxic pathway in teleost CD8$^+$ T cells, no evidence has been reported on its presence.

The ginbuna crucian carp is the only fish species that offers many opportunities for both *in vivo* studies, e.g. graft-versus host reaction, GVHR (Nakanishi and Okamoto, 1999); adoptive transfer of immunity (Nakanishi, 1987; Somamoto et al., 2002) and *in vitro* analysis of CMC using cell lines (Somamoto et al., 2002, 2006, 2009). Furthermore, functional analysis of CD8α$^+$ cells is facilitated by the availability of a monoclonal antibody (MAb) against the CD8α molecule. Recently, three isoforms of the perforin gene have been identified in ginbuna (unpublished data). Ginbuna perforin1, perforin2 and perforin3 were quite different each other, i.e. ginbuna perforin1 shared 34.2% and 35.9% amino acid identity with perforin2 and erforin3, respectively, and perforin2 and perforin3 shared 36.6% amino acid identity in their coding regions. However, basic structures were well conserved as in mammalian and Japanese flounder perforin, i.e. all three isoforms were composed of membrane attack complex/perforin domain and calcium binding domain together with cysteine residues. The characterization of three isoforms of perforin gene will be described more in detail elsewhere.

Here, in order to investigate the role of perforin-mediated pathway in specific cytotoxicity in fish, we evaluated the killing mechanism of ginbuna CD8α$^+$ lymphocytes in the presence and absence of the perforin inhibitors and also analyzed the expression of perforin mRNA after activation by allo-grafting.

2. Materials and methods

2.1. Fish

Ginbuna crucian carp from the island of Okushiri (OB1 clone) were used in this study. Fish, weighing 18–22 g, were maintained in 800 L tanks with running water at a temperature of 25 ± 1 °C and fed twice daily with commercial pellets.

2.2. Cell lines

Three isogeneic fibroblast cell lines, CFS, CFK and CFO that have been established from fin tissue of ginbuna crucian carp from Lake Suwa (S3N clone), Lake Kasumigaura (K1 clone) and the island of Okushiri (OB1 clone), respectively (Hasegawa et al., 1997, 1998), were used in the present study. All cell lines were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Japan) containing 10% fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY) at 30 °C.

2.3. Monoclonal antibody (MAb)

MAb against ginbuna CD8α (2C3 clone) was produced in rat according to the method of Akashi et al. (2003). In brief, CD8α-expressing rat NRK cells were injected into the footpad of a syngeneic Wistar rat. Three weeks after immunization, the animal was sacrificed and lymph node cells were collected and fused with mouse myeloma cells (P3X63-AG8.653). Concerning the specificity of the MAb, magnetically sorted MAb 2C3$^+$ cells are morphologically lymphocytes but not reactive with sIg$^+$ B cells. FACS analysis revealed that MAb 2C3 or 6D1 (CD4 clone) reacted with distinct lymphocyte subpopulations. MAb 2C3$^+$ cells expressed CD8α and TCRβ transcripts, whereas CD8α transcripts were not detected in MAb 2C3$^-$ cells. In contrast, CD4 and IgM transcripts were detected in MAb 2C3$^-$ cells. Specificity of the MAb 2C3 along with specific cytotoxicity against allogeneic target cells by CD8α$^+$ cells has been reported in our previous paper (Toda et al., 2009).

2.4. Sensitization of effector cell donors

To induce CMC, allogeneic sensitization was performed three times as described previously (Toda et al., 2009). The first sensitization was conducted by allogeneic scale grafting, followed by two injections with an allogeneic cell line at 7-day intervals. The CFS cell line was used as the allogeneic cell line, while OB1 clone fish were the effector cell donors. CFS cells were detached with 0.1% trypsin (Difco Laboratories, Inc., Detroit, MI) in phosphate buffered saline (PBS), pH 7.2, containing 0.02% ethylene-diaminetetraacetic acid disodium salt (EDTA) and then washed twice with PBS. Prior to immunization, fish were anaesthetized with 35 ppm ethyl-4-aminobenzoate (Benzocaine, Sigma, St. Louis, MO). Ten scales from a S3N clone fish were transplanted into each OB1 clone fish. Fish were given intravenous injection of 1×10^5 cells/g fish in 0.1 ml of PBS.

2.5. Preparation of effector cells

Preparation of effector cells followed the method of Toda et al. (2009). Briefly, the sensitized (effector donor) fish were anaesthetized and effector cells were aseptically harvested from kidneys (head- and trunk-kidney) 7 days after the final sensitization. Kidney effector cells were disaggregated by pressing through a 150-gauge mesh stainless steel sieve in OPTI-MEM (GIBCO-BRL, Grand Island, NY). The cells were washed with OPTI-MEM and layered on a Percoll density gradient of 1.08 g/ml followed by centrifugation at 450 × *g* for 30 min at 4 °C. Cells at the interface were collected and washed three times with medium, adjusted to 1×10^7 cells/ml and incubated with $1{:}10^4$ diluted rat anti-ginbuna CD8α MAb (mouse ascites) for 45 min on ice. The cells were then washed three times with medium, adjusted to 1×10^8 cells/ml, and incubated for 15 min at 4 °C with 1 ml of a 1:5 dilution of magnetic bead-conjugated goat anti-rat IgG antibody (Miltenyi-Biotec, Bergisch Gladbach, Germany). After incubation the cells were washed three times with medium and then CD8α-positive and -negative cells were separated with magnetic activated cell sorting (MACS) by applying the cell suspension to a plastic column equipped with an external magnet. The CD8α$^+$ cell fraction was collected and viability was confirmed to be greater than 95% by trypan blue dye exclusion. The CD8α$^+$ fraction was re-suspended in OPTI-MEM supplemented with 10% heat-inactivated FBS (OPTI-MEM-10) and pre-cultured for 12 h at 25 °C in a humidified atmosphere with 5% CO_2 in order to enhance the activity of effector cells (Suzumura et al., 1994).

2.6. Preparation of target cells

The allogeneic cell line used as immunogen, CFS was employed as target cells in cytotoxicity assay. In addition, the syngeneic cell line, CFO, and a third party allogeneic cell line, CFK were also used as control cells. Target cells were labeled with CFSE (Molecular Probes, Eugene, OR) for detection by flow cytometry. Target cell suspensions were adjusted to 2×10^6 cells/ml and labeled with 5 μM CFSE for 15 min at room temperature. The reaction was stopped by the addition of an equal volume of FBS at 4 °C followed by three washes.

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