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Identification of a novel fish granzyme involved in cell-mediated immunity

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ABSTRACT

Granzymes (Gzms) are serine proteases released from cytoplasmic granules within cytotoxic T lymphocytes and natural killer (NK) cells. Gzms induce apoptosis within virus-infected and transformed cells. In fish as well as mammals, Gzms appear to play a major role in inducing target cell death. However, information on the function of fish Gzms is limited, although Gzm-like genes have been reported in several species. We identified and characterized a fish Gzm (termed gcGzm) in ginbuna crucian carp, *Carassius auratus langsdorfii*. The primary structure of gcGzm resembled mammalian GzmB, and gcGzm clustered with mammalian GzmB by phylogenetic tree analysis. gcGzm was secreted from HEK293T cells transfected with *gcgzm* cDNA and was predominantly expressed in $CD8^+$ T cells, as in mammals. Expression of *gcgzm* mRNA was greatly enhanced by allo-sensitization and infection with the intracellular pathogen *Edwardsiella tarda*, indicating that gcGzm is involved in cell-mediated immunity. However, its enzymatic activity was different from mammalian Gzms because gcGzm did not cleave the known substrates for mammalian Gzms. Thus we conclude that the newly discovered gcGzm is a novel secretory serine protease involved in cell-mediated immunity in fish, with similar structure to human GzmB but different substrate specificity.

1. Introduction

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells play crucial roles in cell-mediated immunity against allografts, tumors, viruses and intracellular parasites. These effector cells kill target cells by secretory and/or non-secretory pathways. The Fas ligand (FasL) and tumor necrosis factor (TNF) are involved in the non-secretory pathway. Engagement of receptors (Fas and TNF-R) with cognate ligands (FasL or TNF) triggers apoptotic cell death, and leads to further signaling within target cells (Nagata and Golstein, 1995). In the secretory pathway, cytotoxic granules are secreted from CTLs and NK cells by target cell recognition. These granules contain the pore-forming protein "perforin" and granule-associated proteases "granzymes". Granzymes (Gzms) invade cytoplasm of target cells via cell membrane disrupted by perforin, and kill target cells by additional signaling pathways (Lieberman, 2003; Trapani and Smyth, 2002).

Gzms belong to a class of serine proteases and are structurally related to chymotrypsin, with a conserved catalytic triad of key residues: histidine, aspartic acid, and serine. They are genetically similar to other leukocyte serine proteases (e.g. mast cell chymase, (Trapani, 2001)). Although a large number of Gzm genes have been reported in mammals, only two (GzmA and GzmB) have been clearly defined with respect to enzymatic activity and function in the cell death pathway (Johnson et al., 2003). The Gzms hydrolyze cellular substrates and induce apoptotic signals. The specific substrates for each Gzm are clearly different. GzmB induces the cleavage and activation of several caspases (e.g. caspase-3, caspase8) and induces caspase-dependent apoptosis (Andrade et al., 1998; Goping et al., 2003). On the other hand, GzmA induces caspase-independent cell death by cleavage and activation of three members of the SET complex (HMG2, Ape1 and SET) (Fan et al., 2003). Cleavage of the SET complex releases the DNase "NM23-H1" from its inhibitor and subsequently nicks chromosomal DNA (Chakravarti and Hong, 2003).

Gzms are classified based on their substrate specificities with synthetic substrates. Four different enzymatic activities have been reported in mammalian Gzms: tryptase (cleaving after Arg or Lys), Asp-ase (cleaving after Asp), chymase (cleaving after Phe, Trp or Tyr), and Met-ase (cleaving after Met) (Hudig et al., 1987; Woodard et al., 1998; Smyth et al., 1993; Odake et al., 1991; Poe et al., 1988, 1991). These activities are matched with the following

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Gzm: tryptase (Gzm A and K), Asp-ase (GzmB) and Met-ase (Gzm M) (Kam et al., 2000). Many of the other Gzms are predicted to have chymase activity (Praveen et al., 2006).

Fish are the most primitive vertebrates that possess an adaptive immune system with lymphocyte subsets. Fish leucocytes are able to execute cell-mediated cytotoxicity against altered and foreign cells. Cytolytic activity has been reported in ginbuna crucian carp (*Carassius auratus langsdorfii*) leukocytes against virus-infected cells (Somamoto et al., 2013), and in cultured channel catfish (*Ictalurus punctatus*) PBL against allogeneic cells (Zhou et al., 2001). Recently, alloantigen specific cytotoxic cells from ginbuna crucian carp have been identified as CD8α^+ T cells with characteristics similar to those of CTLs in mammals (Toda et al., 2009). In addition, non-specific cytotoxic cells (NCC) have been reported in various fishes (e.g. rainbow trout (*Onchorhynchus mykiss*) (Greenlee et al., 1991)) and NK-like cells have been demonstrated in channel catfish (Shen et al., 2004).

The perforin–Gzm pathway has been reported to play a major role in inducing target cell death in ginbuna crucian carp. The cytotoxicity of allo-antigen specific CD8$^+$ T lymphocytes was lowered by inhibition of the perforin/GzmB pathway (Toda et al., 2011a,c). Perforin mRNA expression and GzmB-like protease activity have been shown to significantly increase by allo-antigen stimulation (Toda et al., 2011a,c).

Although Gzm genes have been identified in some fishes (e.g. carp (Companjen et al., 2006), tilapia (Praveen et al., 2006), salmon (*Salmo salar*), channel catfish and cod (*Gadus morhua*) (Wernersson et al., 2006)), information on their function, role in cell-mediated immunity, and enzymatic characteristics is limited.

In the present study, we identified and characterized a novel fish Gzm. We found that ginbuna crucian carp Gzm (termed gcGzm) was expressed predominantly in CD8$^+$ T cells and expression was greatly up-regulated after allo-sensitization and/or infection with the intracellular bacterium *Edwardsiella tarda*. We also showed that gcGzm is a secretory molecule, as in mammals. However, the substrate specificity was different from those of mammalian Gzms. These results suggest that gcGzm is a novel Gzm with enzymatic characteristics different from those of mammalian Gzms and is involved in cell-mediated immunity in fish.

2. Materials and methods

2.1. Fish

Two isogeneic clones of ginbuna crucian carp from Lake Suwa (S3N clone) and the island of Okushiri (OB1 clone) were used. 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

The Ginbuna crucian carp cell lines CFS and GTS9 were established from fin tissue and thymus of an S3N clonal ginbuna, respectively. These cells were maintained in Leibovitz's L-15 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY) at 30 °C. HEK293T cells (Human Embryonic Kidney 293 T cells) were maintained in RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% FBS at 37 °C under 5% CO_2.

2.3. Monoclonal antibodies

Monoclonal antibodies (MAbs) against ginbuna CD8α and CD4 were produced as per the method reported by Akashi et al. (2003) and the characteristics of the MAbs have been described in our previous papers (Toda et al., 2011a; Toda et al., 2011b). A MAb against ginbuna IgM was produced in mice by injecting purified ginbuna IgM following standard protocols and has been used to separate sIgM$^+$ cells and sIgM$^-$ cells in our previous papers (Somamoto et al., 2006; Takizawa et al., 2008).

2.4. Bacteria

The intracellular fish pathogen *E. tarda* was used in the present study. *E. tarda* was kindly supplied by Dr. Mano, Marine Biotechnology Lab., Nihon University, Japan. *E. tarda* was grown at 25 °C in tryptic soy broth for 48 h, and then cells were harvested by centrifugation at 1,000*g* for 10 min at 4 °C. Harvested *E. tarda* was washed with and then re-suspended in phosphate buffered saline (PBS, pH 7.4) for inducing *E. tarda* infections as described below.

2.5. Isolation of gcgzm cDNA

Total RNA from ginbuna crucian carp thymus was extracted using an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The synthesis of cDNA was performed using the FirstChoice RLM-RACE Kit (Life Technologies) according to the manufacturer's protocol. To obtain a partial cDNA of *gcgzm*, PCR was performed using primers (Table 1) that were designed based on the sequence of the zebrafish *gzm b*-like gene (GenBank Accession Number LOC100003531). The reactions were carried out in 50 μl mixtures containing PrimeSTAR HS DNA polymerase (TaKaRa Bio, Shiga, Japan), with reaction conditions consisting of denaturation at 96 °C for 2 min and 30 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 10 s, polymerization at 72 °C for 30 s, and extension at 72 °C for 2 min. The PCR product was subcloned into the pGEM-T Easy vector using the TA-Cloning method (Promega, Madison, WI). A cDNA fragment coding for the full length of *gcgzm* was generated by 5′-RACE and 3′-RACE PCR using the FirstChoice RLM-RACE Kit and specific primers designed from partial sequences of *gcgzm* (Table 1), according to the manufacturer's protocol. These fragments were cloned into the pGEM-T Easy vector, and the cloned nucleotide sequence was determined using a DNA sequencer (ABI 3100; Applied Biosystems, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

2.6. Preparation of allogeneic sensitized lymphocytes

The first sensitization was conducted by allogeneic scale grafting. Ten scales from a S3N clone fish (donor) were transplanted into OB1 clone fish (recipients). After 7 days, the CFS cell line was injected two times at 7-day intervals, as the second and third allogeneic sensitizations. CFS cells grown in 10 cm dishes were scraped with a cell scraper (Corning Incorporated, Corning, NY), and the cells were collected and washed twice with PBS. The OB1 clone recipients were given i.p. injections of 1×10^5 cells/g fish in 0.1 ml of PBS. Seven days after the final sensitization, the fish were anesthetized with benzocaine, bled from the caudal vessels, and euthanized. Kidneys and spleens were disaggregated by pressing through a 150-gauge stainless mesh steel sieve in Hank's Solution Nissui Pharmaceutical Co.) supplemented with 0.2% heat-inactivated FBS. The cells were washed with this medium, laid on a Percoll (GE healthcare) density gradient of 1.08 g/ml and centrifuged at 450*g* for 30 min at 4 °C. The cells at an interface were collected and washed three times with the medium. Cells were adjusted to 1×10^7 cells/ml and incubated with 1:1000 diluted antibodies against ginbuna CD8α, ginbuna CD4, or ginbuna IgM (mouse ascites) for 1 h at 4 °C. The cells were then washed three times with the medium, adjusted to 1×10^8 cells/ml, incubated for 30 min at 4 °C with diluted (1:10) magnetic bead-conjugated goat anti-rat IgG or anti-mouse IgG antibody (Miltenyi Biotec, Bergisch-Gladbach,