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Effects of IFNγ administration on allograft rejection in ginbuna crucian carp

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ABSTRACT

In vertebrates, the rejection of allografts is primarily accomplished by cell-mediated immunity. We recently identified four IFNγ isoforms with antiviral activity in ginbuna crucian carp, *Carassius auratus langsdorfii*. However, involvement of the IFNγ isoforms in cell-mediated immunity, especially in T cell function remains unknown. Here we investigate expression of the IFNγ isoforms and effects of administration of recombinant IFNγ (rgIFNγ) isoforms in ginbuna scale allograft rejection. All four IFNγ isoforms showed significantly higher expression with the progression of graft rejection. Administration of rgIFNγrel 1 but not rgIFNγrel 2, rgIFNγ1 nor rgIFNγ2 enhanced allograft rejection. The number of $CD4^+$ and $CD8\alpha^+$ cells increased in early stages of rejection, while $sIgM^+$ cells were higher than controls at day 0 and 5 in the rgIFNγrel 1 administrated group. Expression of IFNγ1 and IFNγ2 mRNA was significantly up-regulated by rgIFNγrel 1 administration, while that of IFNγrel 1 and IFNγrel 2 was not. These results suggest different contributions of the four IFNγ isoforms toward the immune responses comprising allograft rejection.

1. Introduction

The immune response in allograft rejection in fish is primarily accomplished by cell-mediated immunity, as in mammals (Nakanishi et al., 2011). In mammals, allogeneic skin graft rejection occurs invariably in an acute manner and is completed within 10–14 days (Benichou et al., 2011b; Cobbold and Waldmann, 1986). The rejection process in mammals is divided into three stages: recognition of alloantigen by T cells, expansion of allo-reactive effector T cells, and effector T cells attacking the allograft (Le Moine et al., 2002). Among the lower vertebrates, acute allograft rejection is documented to be completed in less than 14 days (McKinney et al., 1981). In fish, scale graft rejection occurred within 7 days (Hildemann, 1957) and appeared as the breakdown of melanophores on the epidermis (Shibasaki et al., 2015). Thus, allogeneic scale graft is comparable to skin graft in mammals. A considerable number of studies have reported that alloantigen specific cytotoxicity is primarily mediated by $CD8\alpha^+$ T cells in fish. For example, Zhou et al. reported the existence of alloantigen specific Cytotoxic T lymphocyte (CTL) by establishing a clonal cell line in channel catfish (Zhou et al., 2001). Fischer et al. reported that a T cell fraction enriched by magnetic depletion of B cells and phagocytic cells expressed CD8 mRNA and showed cell-mediated cytotoxicity (CMC) against allogeneic cells (Fischer et al., 2003). We previously demonstrated that $CD8\alpha^+$ T cells but not $CD4^+$ T cells and surface (s)IgM^+ cells showed alloantigen specific cytotoxicity in ginbuna crucian carp (Toda et al., 2009). Furthermore, we documented sequential infiltration of leukocyte subpopulations during allograft rejection (Nakanishi et al., 2015; Shibasaki et al., 2015). Briefly, $CD4^+$ T cells first infiltrated allogeneic scales, followed by $CD8\alpha^+$ T cells and $sIgM^+$ cells, and finally phagocytic cells, suggesting that T cell subpopulations play crucial roles and work together with other cell types for completion of acute allograft rejection.

Numerous studies have revealed that expression of IFNγ is associated with the progression of graft rejection in mammals. For example, Mottram et al. reported that in the normal rejection process, graft-infiltrating T cells produced IL-2 and IFNγ. In contrast, accepted allografts showed low levels of IL-2 and IFNγ

Abbreviations: CMC, cell-mediated cytotoxicity; CTL, cytotoxic T lymphocyte; sIgM, surface IgM.

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expression by leukocytes in their heart transplantation model (Mottram et al., 1995). Landolfo et al. demonstrated that neutralization of IFNγ resulted in suppression of allograft rejection (Landolfo et al., 1985). Both NK cells and T cells are known to produce IFNγ (Boehm et al., 1997). During allograft rejection, activated NK cells produce IFNγ (Benichou et al., 2011a) and enhance antigen-specific T cell proliferation and IFNγ production (McNerney et al., 2006).

In addition to IFNγ, homologous to that of mammals, fish specific IFNγ called IFNγrel were identified in some teleosts (Aggad et al., 2010; Chen et al., 2009; Grayfer and Belosevic, 2009; Milev-Milovanovic et al., 2006; Peng et al., 2015; Shibasaki et al., 2014). Notably, a considerable number of studies reported a functional difference between IFNγ and IFNγrel. For example, differential mRNA expression of carp IFNγ and IFNγrel was detected in T cell and B cell enriched population after *in vitro* stimulation with LPS and PHA (Stolte et al., 2008). Additionally, different gene expression pattern was observed in recombinant IFNγ and IFNγrel stimulation. For instance, Grayfer et al. reported that IFNγrel 2 (they called IFNγrel in their report) stimulation caused a significant down-regulation in the expression of $p40^{phox}$, whereas IFNγ had no effect on the expression of this gene in *in vitro* stimulated goldfish monocytes (Grayfer et al., 2010). Arts et al. reported that expression of CXCb was increased by IFNγ but not by IFNγrel 2 stimulation in carp head kidney phagocytes (Arts et al., 2010). The involvement of IFNγ in cell-mediated immunity has also been reported in fish. In rainbow trout, IFNγ up-regulated the expression of MHC class II mRNA in a macrophage cell line (Zou et al., 2005). Grayfer et al. reported that recombinant IFNγ and IFNγrel stimulated phagocytic and bactericidal activity of macrophages in goldfish, *Carassius auratus* (L.) (Grayfer et al., 2010). Araki et al. reported that kidney leukocytes co-cultured with allogeneic cell lines showed higher expression of IFNγ mRNA compared to isogeneic co-cultivation, suggesting the involvement of IFNγs in cell-mediated immunity (Araki et al., 2013). However, the role of IFNγ particularly in T cell function remains unknown.

Recently, we reported the existence of four IFNγ isoforms, IFNγ1, IFNγ2, IFNγrel 1 and IFNγrel 2, in ginbuna crucian carp and observed that all of four interferons exhibit anti-viral activity (Shibasaki et al., 2014; Yabu et al., 2011). However, the roles of each IFNγ isoform in cell-mediated immunity remain unknown. In the present study, we examined the expression of the IFNγs and the effect of recombinant ginbuna IFNγ (rgIFNγ) administration in our scale graft model. Administration of rgIFNγrel 1 enhanced allograft rejection and increased the number of $CD4^+$ and $CD8\alpha^+$ cells at an early stage of rejection. Expression of IFNγ1 and IFNγ2 mRNA was significantly up-regulated by rgIFNγrel 1 administration. These results suggest the differential contribution of four IFNγ isoforms to allograft rejection providing new insight into the mechanisms regulating allograft rejection mediated by IFNγ.

2. Materials and methods

2.1. Fish

Triploid clonal ginbuna crucian carp (*Carassius auratus langsdorfii*) from Okushiri Island (OB1) and Lake Suwa (S3N) were used as donors and recipients, respectively. Fish weighing 15–20 g (1 year old) were maintained in tanks with running water at 25 ± 1 °C and fed twice daily with commercial pellets throughout the experiments.

2.2. Recombinant protein production

Expression and purification of recombinant IFNs were performed as previously reported (Yabu et al., 2011; Shibasaki et al., 2014). After the three chromatography purification steps (sequential His-tag affinity purification, gel filtration chromatography, and EndoTrap Red (Cambrex Bioscience, MD, USA) endotoxin-removal), the recombinant proteins were used for *in vivo* administration. Removal of lipopolysaccharide was confirmed using a Limulus ES-II Single Test (Wako, Osaka, Japan).

2.3. Monoclonal antibodies against ginbuna crucian carp CD4, CD8α and IgM molecules

Flow cytometric analysis used the previously reported rat mAbs 6D1 and 2C3 recognizing ginbuna crucian carp CD4 and CD8α, respectively, and mouse mAb B12 recognizing ginbuna IgM (Shibasaki et al., 2010; Toda et al., 2009, 2011).

2.4. Scale grafting

Donor OB1 and recipient S3N fish were anaesthetized with 35 ppm ethyl-4-aminobenzoate (Benzocaine, Sigma, St. Louis, MO, USA). A total of twelve scales from a donor fish were transplanted above (six scales) and below (six scales) the lateral line of a recipient fish. After transplantation, scales were observed over time with an Olympus SZX12 stereomicroscope with Olympus DP73 digital camera and software (Olympus, Tokyo, Japan).

2.5. Administration of recombinant interferons

In our preliminary experiments, administration of rgIFNγ isoforms was most effective when injected with 0.1 μg/g in 24 h before subsequent treatment. Accordingly, 24 h before allografting, recipient S3N fish were injected 100 μl of PBS or PBS containing 0.1 μg/g body weight of one of the recombinant interferons into the abdominal cavity.

2.6. Preparation of cell suspensions from epidermis

S3N recipient fish were anaesthetized, bled from the caudal vessels, and euthanized. Grafted scales and surrounding epidermis was harvested at various time intervals after grafting and incubated with PBS containing 1 mM DTT (Wako Chemicals, Osaka, Japan) and 1 mM EDTA for 15 min. After incubation, the tissues were washed, minced with scissors and dissociated by incubating with calcium- and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS) containing 0.1 mg/ml collagenase (Wako Chemicals), 0.1 mg/ml DNase (Sigma) and 5% FBS for 90 min with shaking. After the digestion, the tissue was further disaggregated by pressing through a 150-gauge mesh stainless steel sieve into OPTI-MEM (Gibco, UK) supplemented with 0.5% FBS (OPTI-MEM-0.5). The cells were then washed once with OPTI-MEM-0.5, layered onto a Percoll density gradient of 1.08 g/ml and centrifuged at 450*g* for 30 min at 4 °C. The cells at the interface were collected and washed three times with the medium.

2.7. Flow cytometry

Cells from recipient epidermis were resuspended in PBS containing 0.5% FBS at a concentration of 1×10^7 cells/ml and incubated with $1:10^4$ diluted rat anti-ginbuna CD4, CD8α or mouse anti-ginbuna IgM mAb (mouse ascites) for 45 min at 4 °C. The cells were then washed three times with buffer, resuspended and incubated for 30 min at 4 °C with 1 ml of a 1:500 dilution of Alexa 488 donkey anti-rat IgG or donkey anti-mouse IgG antibody (Life Technologies). The cells were washed an additional three times and then suspended in 0.5 ml of PBS with 2.5 μg/ml propidium iodide (Life