Developmental and Comparative Immunology 35 (2011) 193–202

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Clonal growth of carp (*Cyprinus carpio*) T cells *in vitro*

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ARTICLE INFO

Article history:
Received 16 July 2010
Received in revised form
17 September 2010
Accepted 17 September 2010
Available online 14 October 2010

Keywords:
Fish
Carp
T cell
T cell clone
T cell receptor
Helper T cell
CD4
αβ T cell
γδ T cell

ABSTRACT

Carp kidney leukocytes co-cultured with a supporting cell layer resulted in the rapid proliferation of various types of leukocytes including immature leukocytes. Expressions of marker genes for multiple blood cell lineages were observed in the primary culture. However, after several passages, the proliferating cells expressed only T cell and macrophage marker genes.

Further RT-PCR analysis revealed that the proliferating cells expressed TCR constant regions (*Cα*, *Cβ*, *Cγ*, *Cδ*), *CD3γ/δ* and *CD4* (*CD4L-1*), but did not express *CD8α* and *CD8β*. Additionally, *in situ* hybridization analysis showed that the majority of proliferating cells expressed *Cα*, *Cβ*, *Cγ*, *Cδ* and *CD4*. Moreover, 5′-RACE sequences of TCR variable regions (*Vα*, *Vβ*, *Vγ*, *Vδ*) revealed that the proliferating cells contained a polyclonal T cell repertoire, and most of the Vα and Vβ sequences were functional, but the *Vγ* and *Vδ* sequences were non-functional with frame shifts and stop codons. Taken together, these results indicate that the proliferating cells after serial passages predominantly contained CD4+ CD8− αβT cells that simultaneously co-expressed non-functional *γδTCR*. To obtain CD4+ αβT cell (helper T cell) clones, single cells were picked up from the bulk culture, seeded into each well of 96-well plates and cultured in the presence of supporting cells and conditioned media. T cell colonies formed from single cells after 2–3 weeks. These colony cells expressed *Cα*, *Cβ*, *Cδ* and *CD4*, and weakly expressed *Cγ*, but did not express *CD8α*, *CD8β* and *CD4L-2*. Taken together, these results indicate that these clonal T cells resemble a subpopulation of mammalian CD4+ helper T cells.

1. Introduction

In teleosts, various leukocyte cell lines have been established from channel catfish (*Ictalurus punctatus*). Catfish peripheral blood leukocytes (PBLs), unlike mammalian leukocytes, easily proliferated without need for exogenous growth factors and feeder cells, and spontaneously immortalized at high frequency (Vallejo et al., 1991). For instance, culturing normal catfish PBLs generally resulted in long-term proliferation of monocyte/macrophages (Miller et al., 1994). Additionally, transient stimulation of catfish PBLs with particular stimulants resulted in the long-term proliferation of T or B cells (Vallejo et al., 1991; Clem et al., 1996; Wilson et al., 1998). Furthermore, allogenic mixed leukocyte cultures resulted in the proliferation of cytotoxic T lymphocytes (CTLs) and NK-like cells (Shen et al., 2002, 2004). These proliferating cells from catfish PBLs were further cloned and characterized well. Now, a number of leukocyte cell lines, including macrophage, T cell, B cell and NK-like cell lines are established (Miller et al., 2006). Studies with the catfish clonal cell lines have led to many advances in fish immunology (Stuge et al., 2000; Zhou et al., 2001; Miller et al., 2006).

Until now, catfish is the only fish species from which functionally distinct leukocyte lines can be readily obtained. Although a number of macrophage-like cell lines have been established from the other fish species including goldfish (Wang et al., 1995), carp (Weyts et al., 1997), Atlantic salmon (Dannevig et al., 1997) and rainbow trout (Brubacher et al., 2000), long-term proliferation of various leukocyte types from these species has been difficult to achieve. However, even from these species, long-term proliferation of various leukocytes was achieved by co-cultivating the cells with a supporting cell layer (Siegl et al., 1993; Ganassin and Bols, 1999; Moritomo et al., 2004). Moritomo et al. (1996, 1998) reported that cultivation of carp kidney leukocytes resulted in the natural formation of a stromal cell layer and several different leukocytes were found to proliferate in contact with the cell layer for more than 1 month. Diago et al. (1998) reported that co-cultivation of rainbow trout kidney leukocytes with a kidney stromal cell line (TPS) resulted in long-term proliferation of multiple leukocytes including immature leukocytes. These results suggested that the feeder cells or supporting cells were required for the growth of various leukocytes *in vitro* from these species.

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doi:10.1016/j.dci.2010.09.007

We previously described a co-culture system for long-term proliferation of carp (*Cyprinus carpio*) kidney leukocytes (Katakura et al., 2009). To do this, we first established adherent cell lines from various tissues of common carp and ginbuna carp (*Carassius auratus langsdorfii*). Then, using some of these cell lines as supporting cells, we developed a culture system in which carp kidney leukocytes rapidly proliferated and successively transferred for many passages. Multiple blood cells, including immature types of leukocytes were found to be developing in the primary culture. However, in the passage culture, T cells grew fastest and continuously proliferated for more than 60 days and 20 passages. Moreover, this culture system could produce colonies from single kidney cells. Single kidney leukocytes were cultured in the presence of the supporting cells and conditioned media (CM). Colonies composed of macrophages (type I colony), T cells (type II colony) and a mixture of macrophages and T cells (mixed type colony) were formed from single leukocytes. These results indicated that our co-culture system selectively supports the proliferation of T cells and macrophages including common progenitors of both type of cells (Katakura et al., 2010). Although the carp leukocytes were not immortalized, long-term proliferation and clonal growth of T cells could be useful for their functional analysis.

This report describes the cloning and analysis of carp T cells. Moreover, it demonstrates that in a fish species, other than channel catfish, clonal growth of T cells can be established.

2. Materials and methods

2.1. Fish

Common carp (*Cyprinus carpio*) weighting 40–60 g were purchased from a commercial farm. The fish were kept at 25 °C in a recirculation system with filtered water disinfected by ultraviolet light and fed with pelleted dry food once a day.

2.2. Leukocytes preparation

Kidney leukocytes were prepared as previously described (Moritomo et al., 2004). In short, kidney (including both head and trunk kidneys) was dissected from anesthetized fish and minced on a stainless steel mesh by forceps in 5 ml of minimum essential medium (MEM). Cells released from kidney tissue were collected by a centrifugation (400 × *g* for 5 min.). After discarding the supernatant, 2 ml of distilled water was added to the cell pellet to lyse erythrocytes and gently mixed several times with a pipette. Subsequently, remaining kidney leukocytes were washed twice by centrifugations with MEM. Finally the cells were dispersed in ERDF medium (Kyokuto, Japan) containing 20% fetal bovine serum and 2.5% carp serum (ERDF20/2.5).

Blood leukocytes were prepared as described previously (Serada et al., 2005). In short, blood was withdrawn from caudal vein from a fish with heparinized syringe, transferred into a capillary glass tubes. After sealing one side with clay, the tubes were centrifuged at 1500 × *g* for 5 min. Then, leukocytes were obtained by cutting the tubes 2 mm below the leukocyte layer and suspended in MEM. After pelleting down of leukocytes by a centrifugation, supernatant was discarded and 0.2 ml of distilled water was added to the cell pellet to lyse erythrocytes. Remaining blood leukocytes were washed twice by centrifugations with MEM and resuspended in ERDF20/2.5.

2.3. Bulk culture of carp leukocytes and collection of conditioned media

Ginbuna carp thymus cell line (GTS9) or ginbuna carp fin cell line (CFS) was used as supporting cells as previously described (Katakura et al., 2009). In short, supporting cells were seeded into new 25 cm^2 culture flasks and cultivated for 2–4 days until a confluent monolayer was formed. Thereafter culture media were discarded, kidney leukocytes (1–2 × 10^6 cells/flask) or blood leuko-

Table 1
Primers used for RT-PCR.

Target genes (accession no.)	Primers (5′ → 3′)	Length	Blood cell type
Carp β-*globin* (D88115)	F: GGCCAGGTGTCTGATCGTG R: ATCAGCCAGGAGCCTGAAG	241 bp	Erythrocyte
Carp *CD41* (AB429305)	F: CGGCCTACCTACTGCCTGAG R: ACCTGCTTTCAGCTGATGTCC	440 bp	Thrombocyte
Carp *mpx*/*mpo* (AB429306)	F: ACCACAGTATACCAGGCTATAATGC R: GGTTCTCAAACCATAACCTGTCC	270 bp	Neutrophil
Carp *M-CSFR*/*csf1r* (AB526448)	F AACTAA AGCTCGGAA AGACTCTGG R CGCAGGAAGTTCAGA AGATCAC	286 bp	Monocyte/macrophage
Carp *IgM* (*Cμ*) (AB194134)	F: TTCTTCCACCACCCCCAC R: GCTGCAATCTTGAATAGGAACTG	353 bp	B cell
Carp *lck* (AB429309)	F: CGTCGGGTGGCTATCAAGAG R: TGAGCTCATCGGACACCAAA	328 bp	T cell
Carp *CD3γ/δ* (DQ340867)	F: CACGGTTGACACAGGAAGCA R: GAGTGACCAGAGCCCCAATG	221 bp	T cell
Carp *CD4 (CD4L-1)* (DQ400124)	F: GCTGTGATGGCTGGTGACTC R: GACCGACCAGGGAATGTTAGA	336 bp	T cell
Carp *CD4L-2* (AB560883)	F: CTGCCCTGTTCTCTTCCTCA R: GGTGGTTTCCCTTTCCTGTT	338 bp	T cell
Carp *CD8α* (AB462509)	F: GATCCAGAACGACCGAAAAC R: TATGGTGGGGACATCGTCTT	205 bp	T cell
Carp *CD8β* (DQ324046)	F: CGGCTCGGAAACTATCACCT R: GGCTGAACTGTCGGAGGATT	318 bp	T cell
Carp *TCRα (Cα)* (AB120623)	F: TGTTTGGCAACCGACTTCAC R: AGCTCATCCATGCTTTGAAAGTCATC	274 bp	T cell
Carp *TCRβ* (Cβ) (AB430330)	F: ACATGGACCCTTGGAGACAA R: GCCAATTTCATCCACTGTGC	269 bp	T cell
Carp *TCRγ (Cγ)* (DQ367842)	F: TGGGCAAAAAAGAGCGAAAC R: GAGTCGGGTGGACATGTTGG	245 bp	T cell
Carp *TCRδ (Cδ)* (AB541473)	F: TCTTACCTCCAGCAATGCACCT R: TCTATGAACCCTGAGGCAGAAG	210 bp	T cell
Carp *β-actin* (M24113)	F: GTACGTTGCCATCCAGGCTGTG R: ACGTCACACTTCATGATGGAGTTGAAG	465 bp	House keeping