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ScienceDirect
Molecular Immunology 45 (2008) 1792–1798
Molecular Immunology
www.elsevier.com/locate/molimm

Grass carp transforming growth factor-β1 (TGF-β1): Molecular cloning, tissue distribution and immunobiological activity in teleost peripheral blood lymphocytes

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Received 6 September 2007; received in revised form 23 September 2007; accepted 27 September 2007
Available online 5 November 2007

Abstract

Transforming growth factor-β1 (TGF-β1) is a potent regulatory cytokine with pleiotropic effects on the immune system. To examine the role of TGF-β1 in fish immunity, the full-length cDNA of grass carp TGF-β1 was isolated from grass carp spleen. The open reading frame of grass carp TGF-β1, 1134 bp in length, encodes a 377 amino acid protein. Tissue distribution study by RT-PCR showed TGF-β1 mRNA was predominantly expressed in the thymus, head kidney and spleen in grass carp tissues. Moreover, the time-course effect of TGF-β1 on peripheral blood lymphocyte proliferation in response to mitogens was evaluated in grass carp. Interestingly, TGF-β1 induced PBL proliferation while it significantly blocked phytohemagglutinin- or lipopolysaccharide-stimulated PBL proliferation, and TGF-β1 mimicked the stimulatory effects of lipopolysaccharide on grass carp MHC I mRNA expression. These results, for the first time, strongly suggest that TGF-β1 plays a functional role in lymphocyte proliferation in fish.

Keywords: TGF-β1; Lymphocyte proliferation; Grass carp

1. Introduction

Transforming growth factor-β (TGF-β), a member of TGF-β superfamily, is a potent regulatory cytokine with a wide variety of cellular functions, including cell proliferation, differentiation, migration and apoptosis under physiological and pathological conditions (Li et al., 2006b). For the different cell types or in the different conditions, TGF-β has stimulatory or inhibitory effects on immune cells (Li et al., 2006a; Strobl and Knapp, 1999). In general, TGF-β inhibits T cell proliferation by blocking interleukin-2 production and cyclin expression (Li et al., 2006b). Meanwhile, it also blocks T helper 1 and T helper 2 effector cell differentiation and downregulates cytotoxic T cell development (Li et al., 2007). In addition, TGF-β also exerts multiple effects on B cells, macrophages, natural killer cells and dendritic cells, including the regulation of chemotaxis, activation and survival of these cells (Cazac and Roes, 2000; Laouar et al., 2005; Strobl and Knapp, 1999).

Three isoforms of TGF-β (1–3) have been identified in mammals, and TGF-β1 is the predominant form in the immune system (Letterio and Roberts, 1997; Marie et al., 2006). In fish, expression of TGF-β has been reported, and different isoforms of TGF-β genes have been cloned in some fish species, mainly for evolutionary study. For example, TGF-β1 gene has been cloned from the rainbow trout, zebrafish, carp and hybrid striped bass (Harms et al., 2000; Kohli et al., 2003; Zhan and Jimmy, 2000). TGF-β2 gene has been isolated in the common carp (Sumathy et al., 1997), and TGF-β3 gene has been isolated from rainbow trout, European eel and Siberian sturgeon (Laing et al., 2000). However, the functional role of TGF-β in fish immune is still largely unclear.

Vast evidence indicates that TGF-β plays a critical immunoregulatory role in mammals. However, despite of the progress in cloning TGF-β gene in fish, little information is available about the TGF-β regulation and function in fish immune system. In present study, we successfully cloned full-length cDNA of TGF-β1 from Chinese grass carp, and examined the tissue distribution of the grass carp TGF-β1. To further characterize TGF-β1 immunoregulatory properties on the grass carp immunity, we isolated peripheral blood lymphocytes (PBLs)

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doi:10.1016/j.molimm.2007.09.027

from grass carp, and investigated the effects of TGF-β1 on lymphocyte proliferation in the presence or absence of mitogens. These findings will shed a light on the role of TGF-β1 in the regulation of immune system in teleost fish.

2. Materials and methods

2.1. Animals

The 1-year-old immature Chinese grass carp (*Ctenopharyngodon idellus*), weighting from 1 to 1.5 kg, was purchased from Chengdu Tongwei Aquatic Science and Technology Company. Fish was held in laboratory for at least 2 weeks before processing.

2.2. Cloning of grass carp TGF-β1 cDNA

Total RNA was isolated from grass carp spleen using TRIZOL Reagent (Invitrogen). Five micrograms of total RNA were reverse transcribed to cDNA using the Supersript II reverse transcriptase (Invitrogen). TGF-β1 cDNA fragment was obtained by PCR amplification with degenerated primers T1 and T2 (Table 1). PCR fragments obtained were cloned into the pTG-19 vector (Generay Biotech, Shanghai, CH) and selected clones were sequenced.

Based on the obtained partial sequence of TGF-β1, gene-specific primers were designed (Table 1) and 3′/5′ RACE was performed using a GeneRacer Kit (Invitrogen). PCR products were gel-purified and subcloned into the pTG-19 vector for DNA sequencing. The cDNA sequence and deduced amino acid sequence of TGF-β1 were analyzed using the BLAST program from NCBI and the ExPASy Molecular Biology server (http://us.expasy.org). The multiple alignments were made using MEGA3.1 program (www.megasoftware.net), and the neighbor-joining phylogenetic tree was constructed by DNAMAN (Lynnon Biosoft, Quebec, Canada).

Table 1
Primer oligonucleotide sequences and their applications

Primer name	Sequence (5′/3′)	Use
T1	AARCGYATYGARGCCATYCG	TGF-β1 partial
T2	ATCCAYTTCCAVYCCAGRTC	TGF-β1 partial
3P1	AGCAGGTTTACCTGTATCAT	TGF-β1 3′-RACE
3P2	AGACCTGGACTGGAAGTGGAT	TGF-β1 3′-RACE
5P1	TGATGATACTGGTAAACCTGCT	TGF-β1 5′-RACE
5P2	CTCGAATGGCCTCAATACGCT	TGF-β1 5′-RACE
T3	AGCAGGTTTACCTGTATCATCA	Gene expression
T4	ATCCACTTCCAGTCCAGGTC	Gene expression
M1	GACAGCAACATAATGAAAGCT	Gene expression
M2	AACCGTTCCATTGCAATCTG	Gene expression
B1	TTGGTGACGAGGC TCAGAGCA	Gene expression
B2	CACCATCACCAGAGTCCATCAC	Gene expression
Oligodt18	TTTTTTTTTTTTTTTTTT GGCCACGCGTCGACTAGTACTTT	Gene expression
AP	TTTTTTTTTTTTTT	TGF-β1 RACE
AUAP	GGCCACGCGTCGACTAGTAC GGCCACGCGTCGACTAGTACGG	TGF-β1 RACE
AAP	GIIGGGIIGGGIIG	TGF-β1 RACE

2.3. RT-PCR

Tissue distribution of TGF-β1 expression was examined using RT-PCR. Briefly, total RNA was isolated from different tissues of grass carp, and reverse transcription was performed using superscript II reverse transcriptase (Invitrogen). All primers (T3 and T4 for TGF-β1 mRNA, M1 and M2 for MHC I mRNA, and B1 and B2 for β-actin mRNA) used in this study are listed in Table 1. PCR products were separated on a 1.5% agarose gel and visualized under UV light. Photographs were taken with the Universal HOOD II-S.N 76s Imaging-system (Bio-Rad).

2.4. Isolation of grass carp PBLs and WST-8 assay

Peripheral blood was obtained from cardiac atrium of grass carp using a heparinized syringe. Blood was then quickly diluted with an equal volume of D'Hanks solution (Sigma–Aldrich), and lymphocytes were isolated by density gradient centrifugation (Histopaque1.083 kg/l, TBD, CH). After centrifugation, the PBLs in the interface were collected, washed and counted in the presence of 0.4% trypan blue. Cells were resuspended in RPM-1640 (Gibco BRL) supplemented with 10% fetal bovine serum (PAA, Haidmannweg, GM). About 1×10^6 cells/well cells were seeded in 24-well plate (Becton Dikinson) with 1-ml complete mediums. After recovering at 27 °C under 5% CO_2 and saturated humidity for 24 h, drug treatment was performed using different test substances, including lipopolysaccharide (LPS) (10 μg/ml) (Sigma–Aldrich), phytohemagglutinin (PHA) (30 μg/ml) (Sigma–Aldrich), and recombination human TGF-β1 (rTGF-β1) (1 ng/ml) (Peprotech). After that, cell proliferation was evaluated using the WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-ni-trophenyl))-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay kit (Beyotime Institute of biotechnology, Haimen, CH). Briefly, 100 μl of the Cell Counting Kit solution was added to the culture medium, and incubated for additional 3 h. The absorbance was determined at 450 nm wavelength with a reference wavelength of 630 nm. In RT-PCR study, cells were washed and dissolved in TRIZOL Reagent for total RNA isolation.

2.5. Statistical analysis

Data from each experiment were expressed as percentage of the control value. Statistical analysis was performed with Student's *t*-test for the comparison between two groups. Multiple group comparison was conducted by one-way ANOVA followed by a Tukey-Kramer multiple group comparisons test using the GraphPad InStat Software (GraphPad Inc., San Diego, CA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Molecular cloning and sequence analysis of grass carp TGF-β1

Using 5′ and 3′ RACE, We have obtained the cDNA sequence for grass carp TGF-β1 (accession number EU099588). The ORF

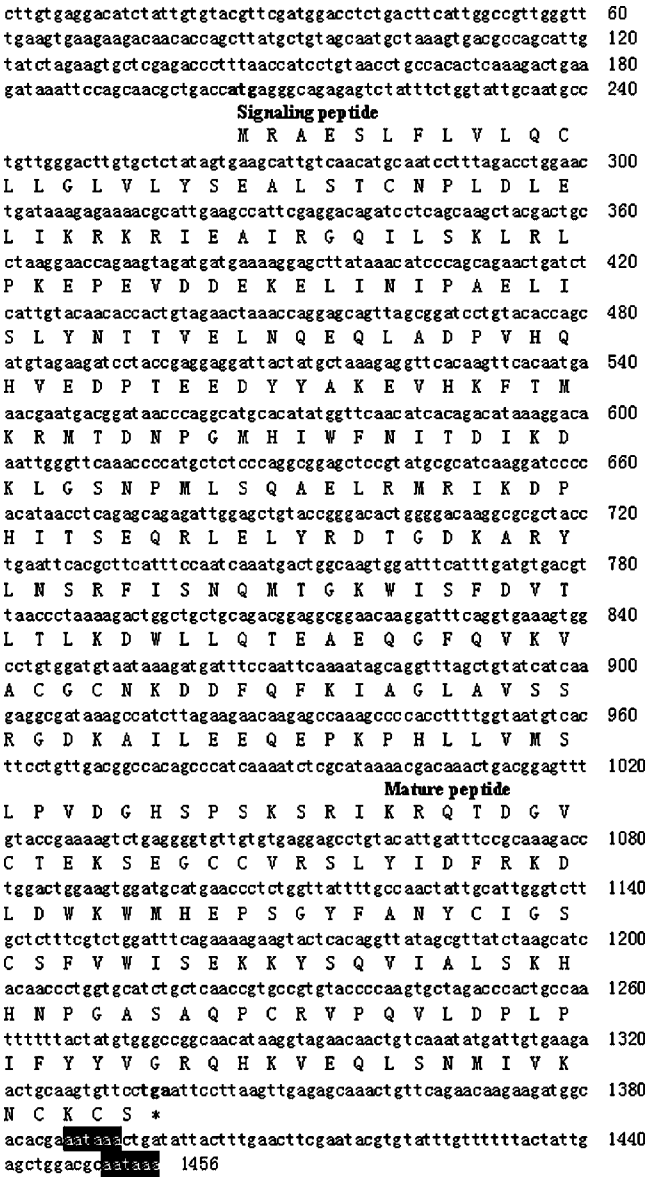

Fig. 1. Full-length cDNA and deduced amino acid sequence of Chinese grass carp TGF-β1. The coding region is calculated using ExPASy. The *asterisk* indicates the stop codon. The putative 3′-UTR polyadenylation signals are marked with black frame.

of TGF-β1 with 1134 bp in length encoded a 377-aa polypeptide including a putative signal peptide and the mature peptide (Fig. 1). TGF-β isoforms have been reported to possess nine invariant cysteine residues to form the "cysteine-knot" which were considered as the distinguishing feature of the TGF-β structure (Harms et al., 2000; Hu et al., 1998; Kohli et al., 2003). Similarly, eight cysteine residues were found in the grass carp TGF-β1 mature peptide and they were conserved in all fish species (Hu et al., 1998; Kohli et al., 2003) (Fig. 2A). Meanwhile, phylogenetic analysis of the amino acid sequence of grass carp TGF-β1 was performed, and the neighbor-joining tree constructed showed two main branches in which all the teleost fish clustered together, while the cluster with mammal TGF-β1 appeared in the other branch (Fig. 2B).

3.2. Tissue distribution of TGF-β1 expression

To examine the tissue expression pattern of TGF-β1 in the grass carp, semi-quantitative PCR was performed using RT samples prepared from selected tissues. To optimize the cycle number for semi-quantitative analysis, PCR amplification profile was examined using the spleen RT sample as the template since TGF-β1 was expressed in the spleen with the highest abundance in other fish species (Harms et al., 2000; Zhan and Jimmy, 2000). Using primers specific for grass carp TGF-β1 (T3 and T4, Table 1), PCR products with 350 bp in size were consistently obtained. The highest level of TGF-β1 transcript expression was detected in thymus, head kidney and spleen, to a lesser extent in pituitary, brain and PBLs, at low levels in heart, kidney and gill (Fig. 3).

3.3. Effects of TGF-β1 on LPS- or PHA-induced lymphocytes proliferation

To test whether TGF-β could affect the responsiveness of PBL to mitogen stimulation (LPS and PHA), the grass carp PBLs were treated with rTGF-β1 (1 ng/ml) supplemented with LPS (10 μg/ml) or PHA (30 μg/ml) or not for 24, 48, 72 and 96 h. WST-8 assay was used to assess the lymphocyte proliferation since it is more reproducible and sensitive than the MTT assay (Miyamoto et al., 2002). Similar to previous studies in mammals, LPS (Fig. 4A) and PHA (Fig. 4B) significantly induced PBL proliferation when treated from 72 to 96 h. However, these stimulatory actions were blocked by rTGF-β1 (Fig. 4A and B).

3.4. Stimulation of TGF-β1 on PBL proliferation and MHC I mRNA expression

To test the direct actions of exogenous TGF-β1 on resting PBL proliferation, a time-course study was conducted by static incubation of PBLs with 1 ng/ml rTGF-β1 for 24, 48, 72 and 96 h, respectively (Fig. 5A). In the parallel experiment, LPS (10 μg/ml) and PHA (30 μg/ml) were used as positive controls in this study because they are effective for stimulating lymphocyte proliferation in other fish species (Ferriere et al., 1996; Reynaud and Deschaux, 2005). Within the first 48 h incubation, rTGF-β1, LPS and PHA did not affect PBL proliferation. But they all induced a time-dependent increase of PBL proliferation from 72 to 96 h when compared with the time-matched controls (Fig. 5A). To further elucidate the immune mechanisms for these effects, the possible involvement of MHC I was examined. In this case, PBLs were incubated with either the control medium or three agents (LPS, PHA and TGF-β) for 96 h and grass carp MHC I mRNA levels were detected by RT-PCR. Results showed a 143 bp PCR product was consistently obtained (Fig. 5B). As expected, LPS (10 μg/ml) markedly increased grass carp MHC I mRNA level compared with the control (Fig. 5B). Meanwhile, rTGF-β1 (1 ng/ml) significantly induced MHC I mRNA expression, whereas PHA (30 μg/ml) had no effect in this regard (Fig. 5B). These results indicated that three stimulators for PBL proliferation might induce immune response through different mechanism pathways.

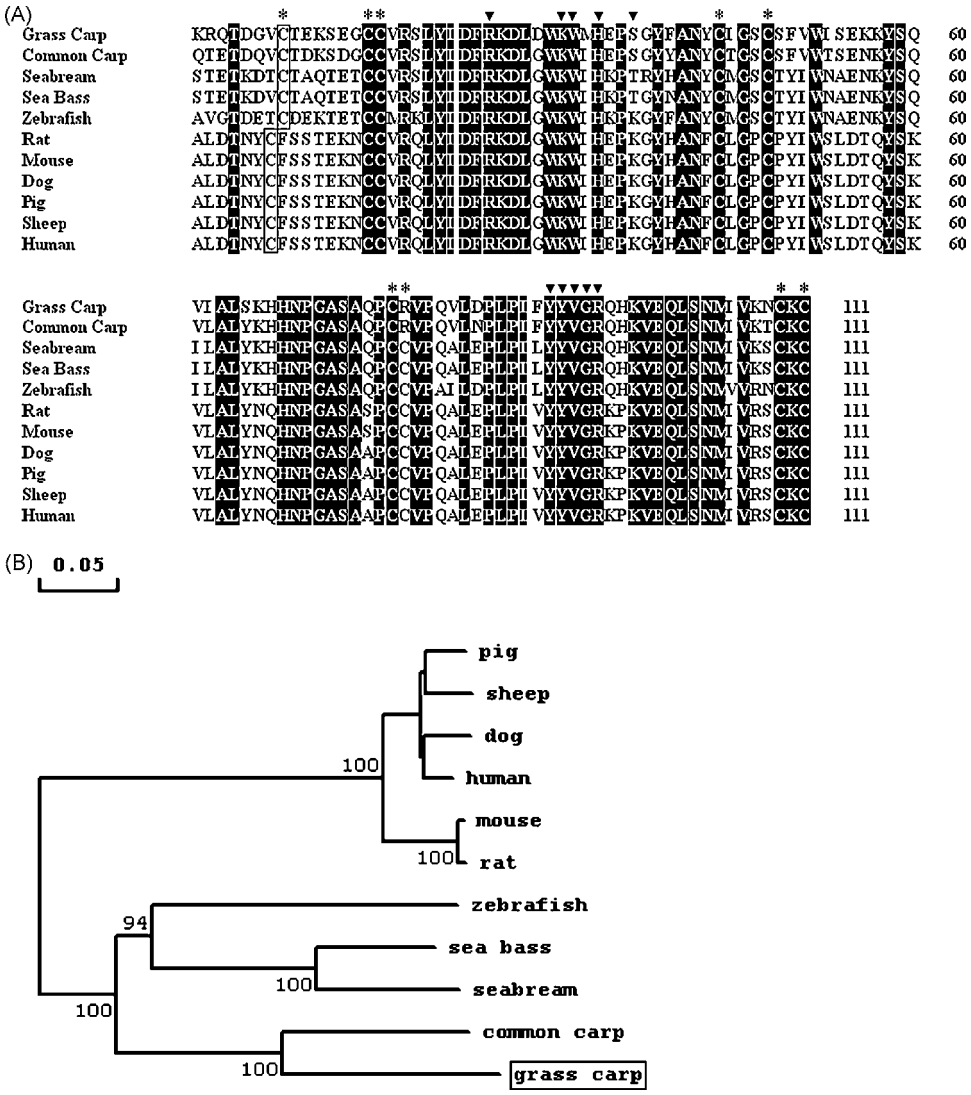

Fig. 2. (A) Multiple amino acid sequences alignment of the known TGF-β1 across different species. The black regions indicate the 100% conserved amino acid residues in all species, whereas the arrows indicate the high conserved amino acid residues in binding regions of TGF-β1 with its receptor. The *asterisks* indicate nine conserved cysteine residues which contributed to form the "cysteine-knot" in mature TGF-β1. GenBank accession numbers are as follows: grass carp, EU099588; common carp, Q9PTQ2; seabream, AAN03842; sea bass, AAD46997; zebrafish, NP878293; rat, NP067589; mouse, NP035707; dog, NP001003309; pig, NP999180; sheep, NP001009400; human, P01137. (B) Phylogenetic analysis of grass carp TGF-β1. The neighbor-joining tree was constructed by DNAMAN based on the amino acid sequences of TGF-β1 available in the GenBank database. The number at each node indicates the percentage of bootstrapping after 1000 replication.

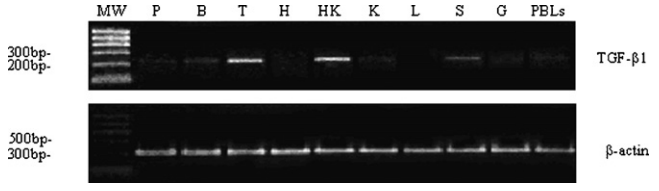

Fig. 3. Expression pattern of grass carp TGF-β1 mRNA in various tissues and PBLs. RT-PCRs were performed using primers specific for TGF-β1 (30 cycles) and β-actin (20 cycles). β-actin was amplified as internal control. P, pituitary; B, brain; T, thymus; H, heart; HK, head kidney; K, kidney; L, liver; S, spleen; G, gill; PBLs, peripheral blood lymphocytes; MW, molecular weight marker.

4. Discussion

The tissue distribution pattern of TGF-β1 has been previously examined in several teleost fish. In common carp (*Cyprinus carpio* L.), TGF-β1 was expressed at low levels in head kidney, spleen, egg and liver, but highly expressed in head kidney leucocytes after activation with concanavalin A (Zhan and Jimmy, 2000). In sea bream (*Sparus aurata*), TGF-β1 expression was also detected in the head kidney macrophages and blood leucocytes (Tafalla et al., 2003). In addition, TGF-β1 mRNA was detected in hybrid striped bass, with higher expression levels in mononuclear cells from peripheral blood than from spleen or anterior kidney (Harms et al., 2000). These studies indicate that distribution of TGF-β1 mRNA has close relationship with fish immunity. In this study, semi-quantitative RT-PCR revealed that TGF-β1 mRNA was expressed in pituitary, brain, thymus, head kidney, kidney, spleen and PBLs in grass carp. Notably, grass carp TGF-β1 was detected to be predominantly expressed in the thymus, head kidney and spleen, which are considered as the major lymphoid organs in fish (Zapata et al., 2006). Actually,

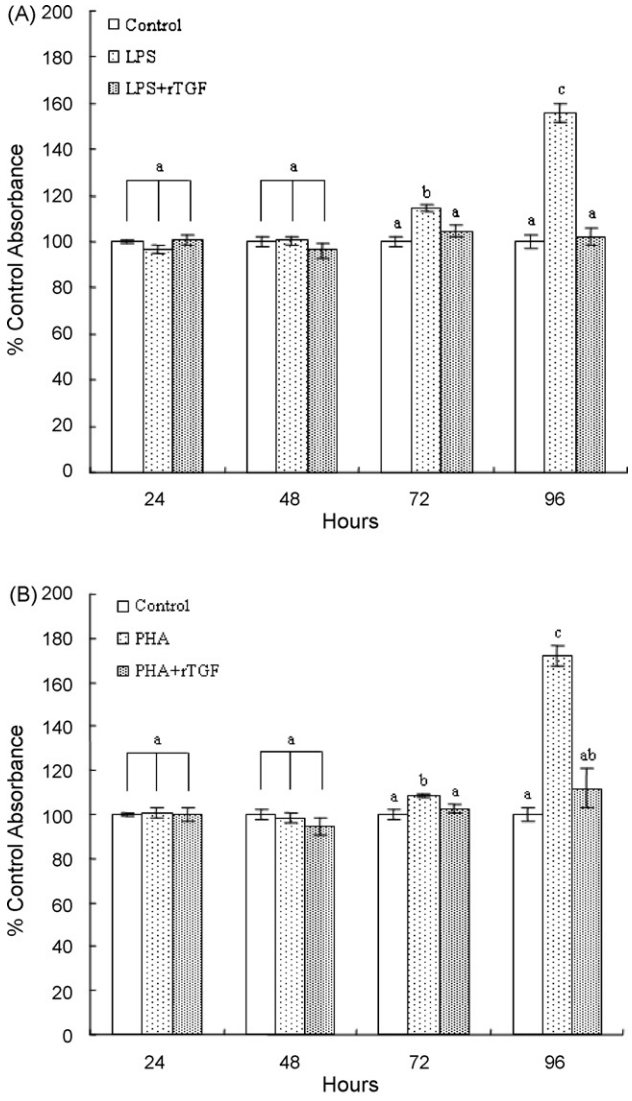

Fig. 4. Effects of TGF-β1 on LPS- and PHA-induced PBL proliferation. The PBLs were incubated for the duration as indicated with LPS (10 μg/ml) (A) and PHA (30 μg/ml) (B) in the presence or absence of rTGF-β1 (1 ng/ml). PBL proliferation was measured by WST-8 assay. Data presented (mean ± S.E.M., $N = 4$) are pooled results from four separate experiments. *Different letters* denote significant difference ($P < 0.05$). LPS, lipopolysaccharide; PHA, phytohemagglutinin; rTGF, recombination human TGF-β1.

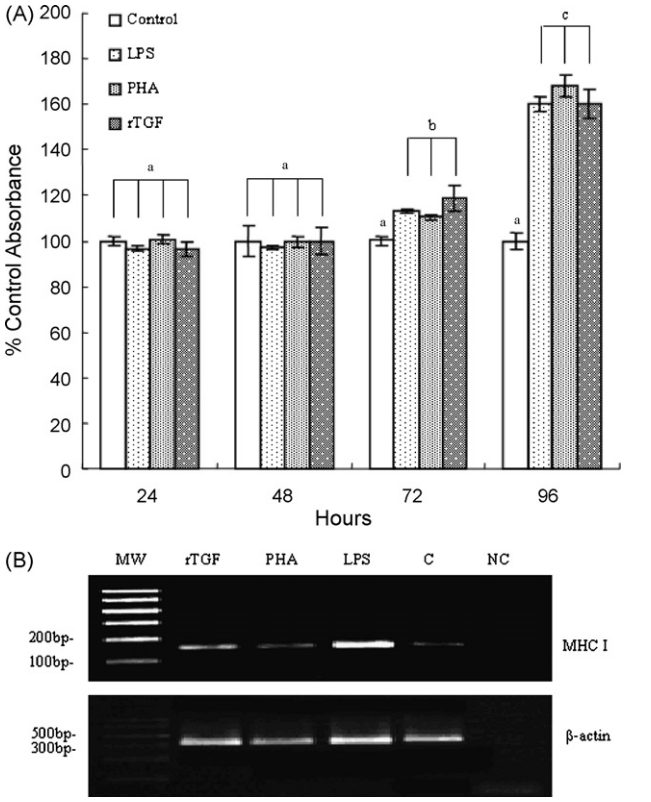

Fig. 5. Effects of LPS, PHA and TGF-β1 on cell proliferation and MHC I mRNA expression in grass carp PBLs. (A) Time-course effects of LPS, PHA and TGF-β1 on lymphocyte proliferation. Grass carp PBLs were incubated for the duration as indicated with LPS (10 μg/ml), PHA (30 μg/ml) and rTGF-β1 (1 ng/ml). PBL proliferation was determined by WST-8 assay. Data presented (mean ± S.E.M., $N = 4$) are pooled results from four separate experiments. *Different letters* denote significant difference ($P < 0.05$). (B) Effects of LPS, PHA and TGF-β1 on MHC I mRNA expression. The PBLs were incubated for 96 h with LPS (10 μg/ml) and PHA (30 μg/ml) and rTGF-β1 (1 ng/ml). RT-PCRs were performed with primers specific for grass carp MHC I (32 cycles) and β-actin (20 cycles). MW, molecular weight marker; LPS, lipopolysaccharide; PHA, phytohemagglutinin; rTGF, recombination human TGF-β1; C, no treatment control; NC, no template control.

the thymus is a primary lymphoid organ responsible for T cell development in teleosts (Boehm and Bleul, 2007). In rainbow trout, developing B cells mature in the head kidney and then migrate to sites of activation, either the spleen or the posterior kidney (Zwollo et al., 2005). It is also reported that the spleen is the first site of IgM expression in cartilaginous fish (Miracle et al., 2001). The highly expression of TGF-β1 in the three major lymphoid organs suggest that TGF-β1 may play a potential role in the immune system of grass carp.

The role of TGF-β as an immunosuppressant factor has been previously examined in mammals, including its antiproliferation activity on T cells and B cells. Studies showed TGF-β has a growth-inhibitory action on T lymphocytes by multiple pathways, including blocking interleukin-2 gene expression (Brabletz et al., 1993), down-regulating the expression of cyclin D2, cyclin E and c-myc (Brabletz et al., 1993; Li et al., 2006b). Similarly, TGF-β is also an important regulator of B lymphocyte activity. For example, TGF-β inhibits proliferation of B cells through the induction of TGF-β target gene Id3, a helix-loop-helix transcription factor (Kee et al., 2001). TGF-β-mediated inhibition of B lymphocyte proliferation also occurs through up-regulation of cyclin-dependent kinase inhibitors (cki) p27 (Bouchard et al., 1997) and down-regulation of c-myc (Massague and Chen, 2000). It is conceivable that an important function for TGF-β inhibition of lymphocyte proliferation is to maintain T/B cell homeostasis and to prevent lymphoproliferative disorder. However, little is known on TGF-β regulation of fish lymphocyte proliferation. To investigate whether TGF-β has a proliferative effect upon fish lymphocytes, grass carp PBLs were isolated and the effect of TGF-β1 on PBL proliferation was evaluated in the presence of PHA or LPS. rTGF-β1 was used in this study because the residues directly involved in the binding of human TGF-β1 with its TβRII were conserved

in grass carp TGF-β1 (Hart et al., 2002) (Fig. 2A). Recent study on zebrafish TGF-β1 also indicated that rTGF-β1 could interact with zebrafish TGF-β1 receptors and activate the downstream signaling pathway (Kohli et al., 2003). Our results showed that rTGF-β1 abolished the PBL proliferation induced by PHA or LPS, which is consistent with the results from mammals that TGF-β1 inhibits lymphocyte proliferation as described above. Thus, we demonstrated, for the first time, that TGF-β1 inhibits in vitro PBL proliferation induced by PHA and LPS in fish model. It is possible that TGF-β1 plays a similar role in controlling fish lymphocyte proliferation as occurs in mammals.

In the present study, the effect of TGF-β1 on lymphocyte proliferation under unstimulated condition was also determined in grass carp. Consistent with the findings in other fish species, both PHA and LPS strongly induced grass carp PBL proliferation. For example, they have been applied to induce PBL proliferation in rainbow trout (Ferriere et al., 1996). Recent studies also employed LPS and another mitogen Con A to induced proliferation of PBL from common carp (Reynaud and Deschaux, 2005). Interestingly, our preliminary time-course studies showed that TGF-β1 also significant stimulated grass carp resting PBL proliferation. To elucidate the immune mechanisms for these effects, the possible involvement of immune-regulating genes were tested at the molecular level. The MHC I was chosen as the target gene for the following reasons: firstly, it is well documented that MHC I is one of the key proteins in specific cell-mediate immune responses, which binds to antigens in the membrane for recognition by lymphocytes through the cell-surface T lymphocyte receptor (TCR) in teleost fish (Fischer et al., 2005; Peatman et al., 2008) as mammals; secondly, the sequences of grass carp MHC I gene recently have been published (Yang et al., 2006) and this allows to study its regulation and role in grass carp immune responses. In this study, the effects of TGF-β1, PHA and LPS on grass carp MHC I mRNA expression were detected. In this case, MHC I mRNA levels markedly increased after LPS treatment for 96 h while PHA at the same time period failed to induce MHC I mRNA expression, indicating that the underlying mechanism might be different for LPS- and PHA-induced immunological responses. Consistent data confirm that LPS induces up-regulation of MHC I molecules in both fish [e.g. seabream (Cuesta et al., 2007)] and mammals [human (Van Overtvelt et al., 2002) and mice (Hobart et al., 1997)], which has been used to mimic inflammatory responses. However, the result for PHA action differs from the report in seabream (Cuesta et al., 2007). Presumably, this discrepancy is due to species-specific and/or cell type variability. In parallel experiments, rTGF-β1 significantly increased MHC I mRNA levels, suggesting that TGF-β1 may induce the antigen-presenting process in grass carp immune response. To our knowledge, the direct stimulatory actions of TGF-β1 on lymphocyte proliferation and MHC I mRNA expression have not been previously reported.

It is also worth mentioning that we demonstrated for the first time that exogenous TGF-β1 stimulated PBL proliferation, which is contrary to findings in mammals as described above. Meanwhile, this result is also at variance with that in actions of TGF-β1 on grass carp stimulated PBLs, suggesting TGF-β1 may play opposite roles in resting and activated lymphocytes in grass carp. The mechanism for such discrepancy is not clear, and the action of TGF-β in immune system is very complex, which depends on many factors, including its concentration, the differentiation states of target cells and the presence of additional regulatory signals from cytokines and co-stimulatory molecules (Li et al., 2006b). In addition, there are noticeable differences in cell subsets of PBL and their differentiation states between mammal and fish, which may contribute to the different results obtained. Whether TGF-β1 in fish acts as a bifunctional factor to be involved in immune regulation as in mammals remains to be determined.

Acknowledgments

This work was sponsored by research grants from Program for New Century Excellent Talents in University (NCEF-06-0814) and the Science and Technology Committee of Sichuan Province. We thank Dr. Yonghua Jiang (University of Pittsburgh, USA) and Dr. Xinyan Wang (University of Hong Kong, Hong Kong) for their advice and assistance.

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