Expressed sequence tags analysis of kidney cells of Japanese flounder, *Paralichthys olivaceus*

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INTRODUCTION

Kidney is an important lymphoid organ in teleost which exhibit a hemopoietic capacity. Evidence supports the presence of antigen-presenting cells, and T-like and B-like lymphocytes in the teleost kidney, and accordingly, a role for the renal lymphoid tissue in the defense mechanisms of teleosts. Antigen-binding cells and antibody-producing cells have been found in the lymphohemopoietic tissue of the teleost kidney.

Expressed sequence tags (ESTs) are short sequences, a few 100 base pairs in length, which are derived by partial, single-pass sequencing of the inserts of randomly selected cDNA clones. Partial sequences of cDNA clones randomly selected from a cDNA library have been shown to be an excellent way of identifying a novel gene, and of characterizing the expressed genes in cell, tissue and species.

In order to make clear the network system of immune and biodefense expressed genes in fish, until now, we have analyzed and reported more than 2,000 clones of expressed genes in the leukocytes of Japanese flounder, *Paralichthys olivaceus*.^{1,2)} In this paper, we performed ESTs analysis of kidney cells of Japanese flounder which is stimulated by LPS and ConA/PMA.

MATERIALS AND METHODS

Sample was taken from kidney of apparently healthy Japanese flounder. Kidney cells cultured in RPMI 1,640 with 10% FBS were stimulated with LPS or ConA/PMA for 1, 3 and 6 hours. The mRNA from each stimulated cell was isolated and used to synthesize double strand cDNA. cDNA libraries synthesis were performed using cDNA synthesis kit (Amersham Pharmacia Biotech, UK) with an oligo dT primer. The cDNA libraries were constructed by ligation to λ ZIPLOX DNA vectors according to the instructions of the manufacturer (Gibco Brl, U.S.A.).

Conversion of the recombinant λ ZIPLOX into the plasmid pZL1 was carried out by *in vivo* excision according to the manufacturer's protocol (Gibco Brl, U.S.A.). The template DNAs for DNA sequencing were prepared in two ways in parallel. One was the alkaline lysis method and the other was the colony direct PCR method using a PCR product pre-sequencing kit (Amersham Pharmacia Biotech, UK). The PCR product pre-sequencing kit was used to prepare the products of symmetric (double-stranded) polymerase chain reaction for sequencing. cDNA clones were sequenced using ThermoSequenase (Amersham Pharmacia Biotech, UK) with M13 forward and/or M13 reverse primers and an automated DNA sequencer LC4,000 (Li-Cor, U.S.A.).

The sequence of each cDNA was compared with reported sequences in the nucleotide and peptide dEST databases of GenBank using the BLAST network service.

RESULTS

Number of clones obtained in cDNA libraries are 5.9×10^5 clones and 1.6×10^6 clones from cells stimulated with LPS and ConA/PMA, respectively. A summary of sequences and clones represented are shown in Table 1. There were 284(76%) of the clones from cells stimulated with LPS and 119 (61%) of the clones from cells stimulated with ConA/PMA revealed homologies with known genes, whereas the remaining 89 (24%) and 79 (39%) clones did not show any significant homology. There were 202 and 82 different genes identified from the cDNA library stimulated with LPS and ConA/PMA, respectively. Total nucleotides that we determined in this study are approximately 229,000 bp and 126,000 bp from cells stimulated with LPS and ConA/PMA, respectively. The average sequenced length was approximately 600 bp.

Clones corresponding to known genes were classified into 6 broad functional categories according to their function and structural motifs, representing cell division, defense and homeostasis,

gene expression, regulation and protein synthesis, metabolism, signaling and communication, and structure and motility. From this data, we found that about 24% were previously reported in fish or have homology with fish genes, whereas 51% of the LPS stimulated and 32% of the ConA/PMA stimulated kidney cells cDNA libraries were new reports.

Table 1. Summary of sequences and clones represented.

Category	Number of clones in each stimulated cells	
	LPS	ConA/PMA
Templates sequenced	373	198
ESTs	373	215
Putatively identified clones	284	119
Unidentified clones	89	79
Putatively identified different genes	202	82
Total nucleotides (bp)	229,278	125,709
Average of sequenced length (bp)	615	585

The genes expressed and the abundance of identified ESTs from cells stimulated with LPS and ConA/PMA were different. The most frequently identified ESTs of cells stimulated with LPS was ubiquitin-like protein, which was prevalent at 2%, while the most frequently identified ESTs of cells stimulated with ConA/PMA was heat shock protein 90 α , which was prevalent at 3%.

Several immune-related genes were induced and expressed by this stimulation, such as CD9, CD45, CD82, fas antigen ligand, interferon inducible Mx protein, immunoglobulin, T-cell receptor, thymosin beta, thioredoxin, MHC class I and II, some transcription factor, some stress induced protein, equistatin precursor and NK-lysin precursor. The last two genes (equistatin precursor and NK-lysin precursor) have never been reported in fish.

DISCUSSION

The number of clones in the constructed cDNA libraries were thought to be sufficient to cover the predominantly expressed mRNAs in stimulated Japanese flounder kidney cells. Clones that correspond to gene expression, regulation and protein synthesis are often found in both of cDNA libraries (25.7% of cells stimulated with LPS and 16.7% of cells stimulated with ConA/PMA), followed by signaling and communication (18.8% of cells stimulated with LPS and 7.91% of cells stimulated with ConA/PMA), and defense and homeostasis (10.7% of cells stimulated with LPS and 16.3% of cells stimulated with ConA/PMA). The most abundant identified ESTs of cells stimulated with LPS was ubiquitin-like protein

which were sequenced 7 clones of 284 identified clones, while the most frequently identified ESTs of cells stimulated with ConA/PMA was heat shock protein 90 α which were sequenced 11 clones of 119 identified clones. Ubiquitin like-protein gene is involved in gene expression, regulation and protein synthesis, while heat shock protein 90 α gene is one type of stress induced protein. The expressed genes, and the frequency of identified ESTs from each stimulated cells were different. As such this suggests that varying stimulation treatments are required for such analysis.

Several immune related-genes which were induced and expressed by these stimulation have been reported in ESTs of leukocytes, liver or spleen of Japanese flounder, whereas both of Equistatin precursor and Natural Killer-lysin (NK-lysin) precursor are new reports in fish. Equistatin is a protein known to inhibit papain-like cysteine proteinases, and also strongly inhibits cathepsin D, protease which expressed in melanoma of tumor cells. This protein is also involved in activity against aspartic proteinases.⁴⁾ NK-lysin is an antibacterial peptide which showed high anti-bacterial activities by killing *Mycobacterium tuberculosis* and tumor cells, but did not display haemolytic activity.⁵⁾ An experiment using stimulation with human interleukin-2 induced synthesis of NK-lysin specific mRNA in a lymphocyte enriched for T and NK cells. This peptide lysed tumor cell line, but did not lyse red blood cells.⁶⁾ This suggests that NK-lysin is a new effector molecule of cytotoxic T and NK cells.

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