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cDNA microarray analysis of interleukin-1β-induced Japanese flounder *Paralichthys olivaceus* kidney cells

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ABSTRACT: Interleukin-1β (IL-1β) cDNA of Japanese flounder was found to consist of 1329 bp, encoded 247 amino acid residues. Among the fish IL-1β in the databases, the one with the highest identity of Japanese flounder IL-1β was that of seabass (62% identity). The expression of IL-1β was induced by treatment with concanavalin A (ConA)/phorbol myristate acetate (PMA) and lipopolysaccharide. The copy number of IL-1β mRNA was increased 30-fold after stimulation with ConA/PMA. Of 871 cDNA on a microarray, 93 genes (10.7%) were up-regulated or down-regulated by IL-1β at 1, 3 and 7 days post-injection. The induced gene expression was highest on day 1 followed by day 3 and day 7. A total of 7% of known and 3.7% of unknown genes of the 871 tested genes were differentially expressed. Of the genes tested, 7.4% were up-regulated and 3.3% were down-regulated. Cytokine genes such as tumor necrosis factor, granulocyte colony stimulating factor and chemokine receptor A were induced in response to IL-1β. Cell surface antigens such as IgM, MHC class I and CD20 receptor were up-regulated. Signal transduction genes such as Toll-like receptor 1 and SH3P2 were also up-regulated. The glucocorticoid receptor and cAMP early repressor were down-regulated in our microarray analysis.

KEY WORDS: **gene expression, interleukin-1β, Japanese flounder, microarray, real-time polymerase chain reaction.**

INTRODUCTION

Interleukin-1β (IL-1β) is an IL-1 cytokine with a beta trefoil structure that is composed of 12 beta sheets,[1] and plays a pivotal role in the inflammatory response as well as in the maturation and proliferation of many immune cell types.[2] Recently, IL-1β cDNA and genes have been cloned from different fish species.[3] Expression of several humoral and cellular factors, production of reactive metabolites, and secretion of hostile molecules have been suggested to depend on the expression of IL-1β in mammals.[4]

DNA microarray technology is the latest technology used to determine the expression profile of many immune related genes of the Japanese flounder in a short period of time. The most important application of microarrays is in the study of differential gene expression in disease and health, and in normal and abnormal physiological and immunological responses.[5] Microarrays, therefore, identify all the genes that are turned on at the site of infection *in vivo*. They can also be used to study the response of a host to challenge with the pathogen such as a cytokine or gene pathways, signaling pathways, and identification of immune gene responses.[5] The microarray analysis would identify cytokine-responsive genes and help develop models for disease investigation.[6] A microarray analysis of IL-1β identified alterations in the expression of multiple transcription factors, cytokines, growth factors and their receptors, adhesion molecules, proteases and signaling intermediates that may contribute to inflammation in arthritis.[7]

In this study, we determined a full length cDNA of Japanese flounder IL-1β. The expression pattern of IL-1β in peripheral blood leukocytes (PBL) following stimulation with either concanavalin A (ConA)/phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) was carried out by real-time polymerase chain reaction (PCR). We investigated the *in vivo* effects of IL-1β on the transcriptional program of the Japanese flounder kidney by using cDNA microarrays to obtain additional insights concerning the effect of this cytokine gene on the immune mechanism of Japanese flounder.

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MATERIALS AND METHODS

Construction of the cDNA library, screening and data analysis

A cDNA library was constructed from the PBL collected from Japanese flounder. The PBL were isolated by centrifugation at 400 ×*g* for 20 min with Percoll solution density gradient (1.072 g/mL). The PBL were cultured at 25°C in RPMI-1640 medium containing LPS (500 μg/mL). mRNA was isolated from PBL incubated in LPS after 1, 3 and 6 h using a micro mRNA purification kit (Amersham Biotech, USA) following the manufacturer's instructions. The purified mRNA of three different time periods were pooled and used to construct a cDNA library. The cDNA library was synthesized using the SUPERSCRIPT plasmid system (Life Technologies, USA) following the manufacturer's instructions for cDNA synthesis and plasmid cloning.

A partial cDNA clone of IL-1β was used as a DNA probe to screen a full length cDNA of IL-1β. The hybridization was conducted as previously reported.[8] The determined nucleotide and deduced amino acid sequences, and multiple sequence alignments were analyzed by GENETYX ver.8.0 (SDC Software Development, Japan). Phylogeny was inferred using the Clustal X and PHYLIP programs, and by distance analysis using the neighbor-joining method.[9] The phylogenetic tree was generated using the Tree view software. The values supporting each node are derived from 100 re-samplings.

Construction of plasmid expressing Japanese flounder-interleukin-1β

pGEM T-Easy vector (Promega, Madison, WI, USA) ligated with the full length Japanese flounder interleukin-1β (JF-IL-1β) cDNA was sub-cloned into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The plasmid was extracted by ultracentrifugation using a CsCl-ethidium bromide gradient.[10]

Quantification of interleukin-1β by real-time polymerase chain reaction

The absolute copy number of the target transcript and standard curve for JF-IL-1β and β-actin were generated as described previously.[11] Briefly, a cloned plasmid DNA for each sample was used to generate a standard curve. The cloned plasmid DNA (0.5 μL) was used in 50 μL of PCR mixture. The PCR primers used in real-time PCR are listed in Table 1. The PCR-reacted products were purified using Amicon Microcon-PCR centrifugal filter devices (Millipore, USA). The copy number of reacted products were calculated according to the molecular weight of the products and then converted into the copy numbers based upon Avagadro's number ($1\ mol = 6.022 \times 10^{23}$ molecules). A total of 50 μL of the PCR reaction was prepared for quantitative real-time PCR. The reaction mixture consisted of 5 μL template DNA (10 μg/mL), 5 μL of both forward and reverse primers (5 μm), 5 μL 10 × SYBR PCR buffer, 6 μL 25 mM $MgCl_2$, 4 μL dNTP blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dUTP), 0.25 μL AmpliTaq Gold (5 U/μL), 0.5 μL AmpErase UNG (1 U/μL) and 19.25 μL distilled water. The PCR amplification was performed as follows: one cycle at 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 58°C for 60 s. Thermal cycling and fluorescence detection was conducted using the Gene Amp 5700 sequence detection system as described above. All samples were run in triplicate with β-actin as an internal positive control[12] and the normalization reference

Table 1 Oligonucleotide primers used for real-time polymerase chain reaction analysis

Name	Sequence (5′-3′)	Information
IL-1Fst (Forward)	5′-cctgctcaacatcatgatga-3′	Designed for making a standard curve in real-time PCR quantitative gene expression analysis of IL-1β and β-actin
IL-1Rst (Reverse)	5′-aagaactttgcggttgctgc-3′	
β-actin Fst (Forward)	5′-tttccctccattgttggtcg-3′	
β-actin Rst (Reverse)	5′-gcgactctcagctcgttgta-3′	
IL-1β Frt (Forward)	5′-cgtctccaccagatcagttcag-3′	Designed for real-time PCR analysis of IL-1β andβ-actin
IL-1β Rrt (Reverse)	5′-gctgttctggaccagaatgagt-3′	
β-actin Fst (Forward)	5′-tgatgaagcccagagcaga-3′	
β-actin Rst (Reverse)	5′-ctccatgtcatcccagttggt-3′	

IL, interleukin; PCR, polymerase chain reaction.

for individual variation. Statistical analysis of expression levels between different samples was analyzed by an independent student's *t*-test. Values were considered significant when $P < 0.05$.

Japanese flounder cDNA microarray construction

An 871 unique element Japanese flounder cDNA microarray was constructed based on the procedures described previously.[13] Briefly, the preparation of the DNA chip was performed as follows. Individual Japanese flounder cDNA clones were carefully chosen to avoid duplication of the same genes from our previous expressed sequence tags (EST) analyses. cDNA clones from EST were used as template DNA and the primers (sense-GTGCTG CAAGGCGATTAAGTTGG, antisense-TCCGGCTCG TATGTTGTGTGGA) were designed to anneal the vector region. The PCR was performed as follows. An initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 2 min, and a final elongation step at 72°C for 5 min. The resulting PCR products were purified and concentrated by a 96-well PCR cleanup kit (Millipore, USA) to attain a final concentration above 500 μg/mL. The purified PCR products were spotted on to the microarray slide by DNA Chip (Research Inc., Tokyo, Japan). β-actin genes were included on the array as controls for labeling, hybridization and fluorescent background.

Fish

In total, 36 Japanese flounder fish, each weighing about 10 g, were used in the study. The fish were maintained in a recirculation seawater system at a constant temperature of 20°C. The fish were divided into two groups and intramuscularly injected with 10 μg of pCMV-JF-IL-1β or pCMV vector, respectively.

RNA preparation for microarray analysis

Total RNA was extracted from both pCMV-JF-IL-1β and pCMV vector kidney tissue of Japanese flounder at 1, 3 and 7 days post-injection, respectively, by TRIZOL reagent (Invitrogen Life technologies, USA) following the manufacturer's instructions.

mRNA was isolated from total RNA using a Quick Prep micro mRNA purification kit (Amersham Biosciences, USA) according to the manufacturer's instructions.

Preparation of fluorescently labeled cDNA and hybridization

A 1 μg of pooled mRNA isolated from pCMV-IL-1β-induced fish and pCMV vector-injected fish were labeled with Cy5-amino-allyl dUTP and Cy3-amino-allyl dUTP, respectively, with a Labelstar array kit (Qiagen, USA) following the manufacturer's instructions. For final probe preparation, a sample containing equal amounts of cDNA labeled with Cy3 or Cy5 was mixed with 7.5 μL of hybridization buffer and 15 μL of formamide, transferred to the microarray glass slide, and incubated at 42°C for 18 h in a custom-made slide chamber in which the humidity was maintained with a few drops of distilled water. After hybridization, the arrays were washed with 2 × standard saline citrate (SSC)-0.1% sodium dodecylsulfate (SDS) for 20 min at room temperature, then washed with 0.2 × SSC-0.1% SDS for 20 min at room temperature, and washed twice with 0.2 × SSC-0.1% SDS for 20 min at 55°C with gentle agitation. After rinsing with 0.2 × SSC-0.1% SDS at room temperature, the slides were dried and scanned immediately using a GenePix 4000B scanner (Axon Instruments, Foster City, CA, USA).

Signal detection and data analysis

The fluorescent intensity for each dye (Cy3/Cy5) was detected with a GenePix 4000B microarray scanner (Axon Instruments). Images were analyzed by GenePix pro 3.0 software provided with the scanner. The signal intensity was normalized to the signal intensity of β-actin by adjusting the PMT power and signal gain. Feature ratios of 2.0 and above were considered as an up-regulation factor and 0.5 and below were considered as a down-regulation factor. GenePix Pro 3.0 displays the data in tables that can be exported to any standard spreadsheet program.

The nucleotide sequence reported for Japanese flounder IL-1β in the present paper has been submitted to the GenBank database and has been assigned the accession number AB070835.

RESULTS

Cloning and analysis of full length cDNA of Japanese flouder-interleukin-1β

The JF-IL-1β cDNA consisted of 1329 bp. The translated open reading frame gave a predicted 247 amino acid precursor peptide with a molecular weight of 28 kDa. The JF-IL-1β cDNA has six

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Jflounder   1:-------------------------MESKMECNVSQMWSAKMPQGLNLEISHHPMTM 32
Seabass     1:-------------------------MESEMKCNMSEMWRSKMPQGLDLEITHHPLTM 32
Trout       1:---- ------------------ MDFESNYSLIKNTSESAAWSSKLPQGLDLEVSHHPITM 38
Carp        1:MACHEYVHQLDLSEAFETDSAIYSDSADSDELDCPDPQSMSCQCDMHDIKLELSSHPHSM 60
Xenopus     1:-MALVPDLSSIPMEGYSGDDEMFYSDSPSGMKDDMEDAAQWQSSTSHCSLDIHVQITHGK 59
Chicken     1:-MAFVPDLD--VLESSSLSEETFYG--PSCLC--LQKKPRLDSE--HTTVDVQVTVRKGR 51
Human       1:-----MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPLDGGIQLRISDH 55
                                                         . .    . ..   ..  .

Jflounder  33:RSVVNLIIAMERLKGSHSESVLSTSFTDENLLNIMMENIVXEHIVCERSSSPPD----Q 87
Seabass    33:RRVVNLIIAMERLKGFSSETLMSTEFRDENLLNIMLESIVEEKIVFERGTTPTA----Q 87
Trout      39:RHIANLI-AMERLKGGEGVT-MGTEFKDKDLLNFLLESAVEEHIVLELESAPPASRRAAG 96
Carp       61:RQVVNIIIAVERLKHIKNMS--SGKFCDEELLGFILENVIEERLVKPLNETPI------ 111
Xenopus    60:GSLHSFRKAVVLVVAVEKL-KR--GKERFFGDEDLLGLLDSIFVEEEIAFSQ-AKETHAS 115
Chicken    52:GA-RSFRRAAVLVVAMTKLLRR--PRSRDFADSDLSALLEEVF--EPVTFQR-LESSYAG 105
Human      56:HYSKGFRQAASVVVAMDKLRKMLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAYVH 115
                   .  . .  * ....      . .  ... ...   ..  .  .    .

Jflounder  88:FSRRG-VYTCNITDSQKRNFI----LVQNSMELHAVMLQGGSSNRKVLLNMSTY---VHP 139
Seabass    88:YSKRR-EVQCSVTDSEKRSLV----LVPNSMELHAVMLQGGSDRCKVQLNMSTY---LDR 139
Trout      97:FSSTS-QYECSVTDSENKCWV----LMNEAMELHAMMLQGGSSYHKVHLNLSSY---VTP 148
Carp      112:YSKTSLTLQCTICDKYKKTMVQSNKLSDEPLHLKAVTLSAGAMQYKVQFSMSTF---VSS 168
Xenopus   116:ASTYRYQRATTCRIKDTSNKCFVMQKFHENAQLVALQLQGANIQREEKVSMAFY---ATQ 172
Chicken   106:APAFRYTRSQSFDIFDINQKCFVLES---PTQLVALHLQGPSSSQKVRLNIALYRPRGPR 162
Human     116:DAPVR---SLNCTLRDSQQKSLVMSG---PYELKALHLQGQDMEQQVVFSMSFV-----Q 164
              ↑  .  .    .  ..        .    .* *  *....   .. .... .

Jflounder 140:SPT-IEARPVVLGIKDTDFFLSC-QK-NGAE-PTLHLERVENKCDLEAFSRDSEMVRFLF 195
Seabass   140:TPS-AEAQTVALGIKGTNYYLSC-HK-DGEE-PTLHLEVVD-KASLANITSDSDMVRFLF 194
Trout     149:VPIETEARPVALGIKGSNLYLSC-SK-SGGR-PTLHLEEVADKDQLKSISQQSDMVRFLF 205
Carp      169:ATQ-KEAQPVCLGISNSNLYLAC-TQLDGSS-PVLILKEAS--GSVNTIKAGDPNDSLLF 223
Xenopus   173:PHQGGSKRPVALGLAGKNLYLSCRATEDGQDSPKLYLEEIS----NIKDVKGEDLNRFIF 228
Chicken   163:GSAGTGQMPVALGIKGYKLYMSC--VMSGTE-PTLQLEEAD----VMRDIDSVELTRFIF 215
Human     165:GEESNDKIPVALGLKEKNLYLSC--VLKDDK-PTLQLESVD----PKNYPKKKMEKRFVF 217
                   .. .*.**... .....*     .    *.* *...             ...*

Jflounder 196:YK--QDSGGVSISTLMSARFPNWYISTS--EQDNRPVMVGQKNAR-CYQTFNIQHQS--- 247
Seabass   195:YK--QDSG-LNISTLTSVPFSNWYISTA--EENNRPVQMCQESAR-RHRAFNIDNLKVDP 248
Trout     206:YR--RNTG-VDISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRLTTFTIQRHN--- 259
Carp      224:FR--KETG-TRYNTFESVKYPGWFISTAFDDWEKVEMNQMPTTRTTNFTLEDQKRI---- 276
Xenopus   229:MKSQDGLNETSTNSFESVAFPGWYISTSQRENELVQMVHQKNQEAIKDFNLFSVI----- 283
Chicken   216:YR-LDSPTEGTTR-FESAAFPGWFICTSLQPRQPVGITNQPDQVNIATYKLSGR------ 267
Human     218:NK----IEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS---- 269
                 ..    .     ...*. ...*.*.*.    .     .

Jflounder 247:-------------                                              247
Seabass   249:TTEDQVCPLLNGQ                                              261
Trout     259:-------------                                              259
Carp      276:-------------                                              276
Xenopus   283:-------------                                              283
Chicken   267:-------------                                              267
Human     269:-------------                                              269
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Fig. 1 Alignment of Japanese flounder interleukin (IL)-1β with sea bass, trout, carp, *Xenopus*, chicken and human IL-1β. Sequences were obtained from the DDBJ/EMBL/GenBank database. Amino acids identical with Japanese flounder are shown by dots. The position of residues identical in all sequences are shown by asterisks. Gaps (dashes) have been placed to maximize identity. The ICE cut site (aspartic acid residue) in mammals is indicated with an arrow.

complete TATTTA sequences and some other incomplete TATTTA sequences in the 3′UTR (GenBank acc. No. AB070835). The identities of the deduced amino acid sequence of JF-IL-1β to other species IL-1β ranging from 27 to 62% (Fig. 1). Japanese flounder IL-1β has the highest peptide similarity (62%) with sea bass, followed by trout with 52% similarity. The amino acid

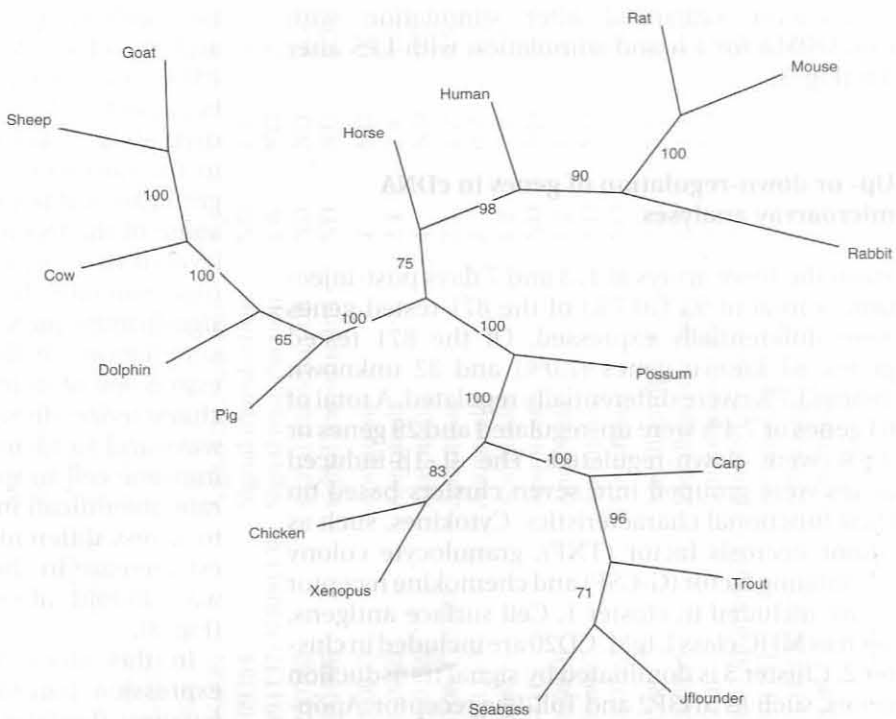

Fig. 2 An unrooted phylogenetic tree showing the relationship between the full length Japanese flounder interleukin (IL)-1β amino acid sequence with other representative IL-1β sequences in different vertebrate groups. The tree was constructed by the neighbor-joining method using the CLUSTAL X and PHYLIP packages, and was bootstrapped 100 times. The sequences were obtained from the DDBJ/EMBL/GenBank database.

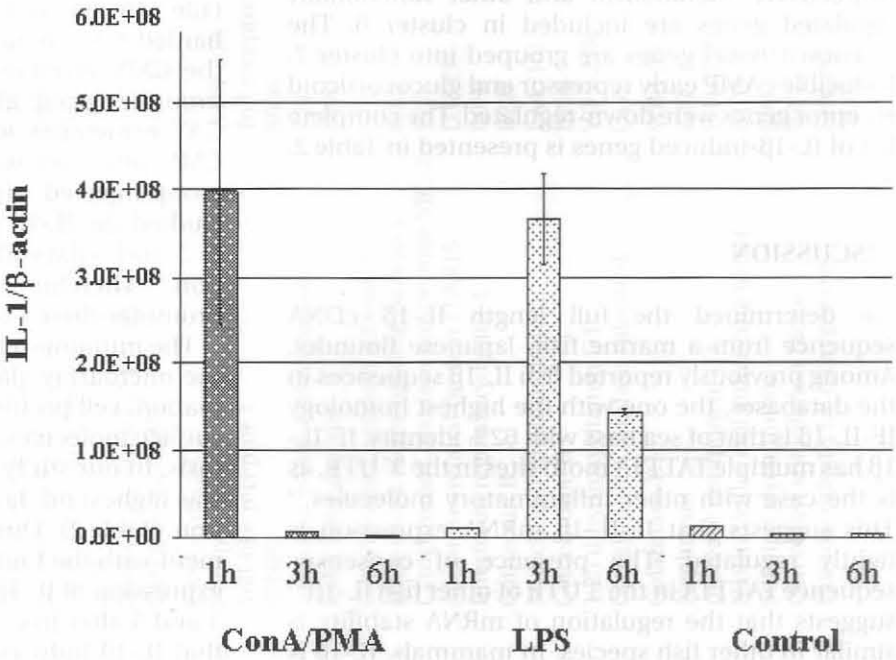

Fig. 3 Quantitative real-time polymerase chain reaction analysis of interleukin-1β expression in Japanese flounder following stimulation of peripheral blood leukocytes with concanavalin A/phorbol myristate acetate or lipopolysaccharide at 1, 3 and 6 h intervals. Standardization was done with the respective β-actin mRNA levels.

sequence alignment (Fig. 1) showed a lack of aspartic acid residues in the region where ICE cuts the mammalian IL-1β precursor (Fig. 1). The phylogenetic tree revealed that the JF-IL-1β branches with sea bass and trout as its nearest neighbors, and mammals are more distant neighbors (Fig. 2).

Quantitative gene expression analysis following stimulation of leukocytes with concanavalin A/phorbol myristate acetate or lipopolysaccharide

As shown in Fig. 3, all stimulated leukocytes were significantly induced as compared to normal leukocytes. The expression of IL-1β mRNA was

significantly enhanced after stimulation with ConA/PMA for 1 h and stimulation with LPS after 3 h (Fig. 3).

Up- or down-regulation of genes in cDNA microarray analyses

From the three arrays at 1, 3 and 7 days post-injection, a total of 93 (10.7%) of the 871 tested genes were differentially expressed. Of the 871 tested genes, 61 known genes (7.0%) and 32 unknown genes (3.7%) were differentially regulated. A total of 64 genes or 7.4% were up-regulated and 29 genes or 3.3% were down-regulated. The IL-1β-induced genes were grouped into seven clusters based on their functional characteristics. Cytokines, such as tumor necrosis factor (TNF), granulocyte colony stimulating factor (G-CSF) and chemokine receptor A, are included in cluster 1. Cell surface antigens, such as MHC class I, IgM, CD20 are included in cluster 2. Cluster 3 is dominated by signal transduction genes, such as SH3P2 and Toll-like receptor. Apoptosis-related genes and inflammation-related genes are included in clusters 4 and cluster 5, respectively. Metabolism and other functionally regulated genes are included in cluster 6. The unknown/novel genes are grouped into cluster 7. Inducible cAMP early repressor and glucocorticoid receptor genes were down-regulated. The complete list of IL-1β-induced genes is presented in Table 2.

DISCUSSION

We determined the full length IL-1β cDNA sequence from a marine fish, Japanese flounder. Among previously reported fish IL-1β sequences in the databases, the one with the highest homology JF-IL-1β is that of sea bass with 62% identity. JF-IL-1β has multiple TATTTA motif sites in the 3′ UTR, as is the case with other inflammatory molecules.[14] This suggests that JF-IL-1β mRNA expression is tightly regulated. The presence of consensus sequence TATTTA in the 3′UTR of other fish IL-1β[3,4] suggests that the regulation of mRNA stability is similar in other fish species. In mammals, IL-1β is produced as an inactive precursor that must be cleaved intracellularly by the IL-1β converting enzyme (ICE).[15] In contrast to this fish IL-1β, precursors cloned to date lack a clear ICE cut site,[4] and this also appears to be the case for the analyzed Japanese flounder cDNA sequence.

Mitogen activation using ConA/PMA generates more lymphocyte clones than does antigenic LPS activation. This is because mitogens carry receptors with different antigen-binding specificities and, therefore, activate more clones. In this study, PMA was used in combination with ConA because ConA and PMA are not mitogenic when used alone.[16] As expected, JF-IL-1β was not found in the non-stimulated leukocytes. In contrast, in gilt head seabream, IL-1β transcript was found in some of the tissues.[17] In our study, the maximum level of IL-1β was observed 3 h after LPS stimulation. Similarly, IL-1β expression in rainbow trout significantly increased between 2.5 and 4 h after stimulation with LPS.[18] Understanding the expression of immune response genes will help to characterize disease-related inflammatory pathways and to identify the functional properties of immune cell subpopulations. Therefore, an accurate quantification of mRNA expression is needed to assess differential gene expression. The greatest increase in the copy number of IL-1β mRNA was 30-fold after stimulation with ConA/PMA (Fig. 3).

In this study, we also focused on the gene expression pattern of immune-related genes of Japanese flounder kidney cells induced by IL-1β *in vivo*, as the kidney is the major lymphoid organ in fish and IL-1β is a potential inflammatory molecule. Tucker *et al.* reported detection of bombarded CAT (reporter gene) expression in fish of the CMV promoter group and fish of the SV-40 promoter group after plasmid DNA injection. The CAT expression in the fish group injected with CMV promoter was higher than that in the fish group injected with SV-40 promoter.[19] Kono *et al.* studied the IL-1β protein expression in serum on 1, 3 and 5 days after pCMV-IL-1β plasmid injection.[20] Therefore, in this study we used a CMV promoter-driven vector.

The immune-related genes that were spotted on the microarray glass slide were related to inflammation, cell proliferation, signaling pathways, cell surface molecules, transcription factors and apoptosis. In our study, IL-1β-induced gene expression was highest on day 1 over days 3 and 7 after injection (Table 2). Our results are in complete agreement with the findings of Kono *et al.*, in which the expression of IL-1β was highest on day 1 over days 3 and 5 after injection.[20] The present results show that IL-1β induced the expression of TNF, G-CSF and chemokine receptor A (Table 2). A typical characteristic of IL-1β is to synergize with a variety of cytokines, including TNF and G-CSF. Synergism between IL-1β and TNF has also been observed *in vivo*.[21] Endotoxin or secondary mediators such as TNF, IL-1β and IFN-γ are major stimulators of G-CSF production *in vivo* and result in rapid but transient elevation in serum.[22] Our results of IL-1β-induced TNF and G-CSF *in vivo* agree with the

Table 2 Interleukin-1β – induced gene expression profile of Japanese flounder kidney cells *in vivo*

Cluster No.	Gene name	Clone Identification	Gene accession No	Function	Expression levels		
					Day 1†	Day 3‡	Day 7§
Cluster 1	Tumor necrosis factor	B894	AU091130	Inflammation	3.4	ND	0.8
(Cytokines)	Granulocyte colony stimulating factor	Y67	AU260798	Granulopoisis	1.2	5	0.7
	CC chemokine receptor A	cc chemoR A	Not submitted	Chemotaxis	1.4	2.7	0.8
Cluster 2	Lymphocyte antigen Ly-6G.1	Y136	AU260850	Cell growth	3.9	4.6	1
(Surface antigens)	Guanine nucleotide binding protein β	L144	AU260989	Receptor coupling	2.7	ND	ND
	MHC class I protein	L7	AU260469	Antigen presenting	2.2	ND	1.1
	CD20 receptor	WF1 (3)	C82121	B cell differentiation	1.4	4.2	0.8
	MHC class I A	WH6-20F	AU050612	Antigen presenting	1.8	3.8	1
	β2-microglobulin	WF9 (1)	C81987	Antigen presenting	1.5	3.5	1.1
	MHC class I antigen	R31	AU260597/AU260890	Antigen presenting	1.4	2.2	1
	Ig M	X43	AU260744/AU260976	Antigen presenting	1.9	2	1
	Immunoglobulin light chain L2	Y143	AU260856	Antigen presenting	1.3	0.8	2
	CD3 gamma/delta	CD3 g/d	AB044572	Antigen presenting	ND	0.5	ND
Cluster 3	Pleckstrin (p47)	WG7 (1)	C81996	Signal transduction	3.3	ND	0.7
(Signal transduction genes)	Phospholipase D	WD8-20R	AU050587	Signal transduction	1.3	5.1	ND
	Toll-like receptor 1	Toll receptor1	AB109394	Signal transduction	ND	1.1	3.2
	SH3P2	B906	AU091137/AU091138	Signal transduction	1.6	ND	2
	Inducible cAMP early repressor	Y24	AU260762	signal transduction	ND	ND	0.5
	KIAA 1064 protein	B620	AU091006	signal transduction	ND	ND	0.3
Cluster 4	Cytochrome C oxidase subunit III	L231	AU260875	Apoptosis	4	ND	0.7
(Apoptosis genes)	Heat-shock factor binding protein 1	WE9-13F	AU050269	Apoptosis	2.4	2.4	1.2
	Cytochrome C oxidase polypeptide VIC-2	Y27	AU260765	Apoptosis	4	ND	0.7
	Apoptosis-related protein TFAR15	LG3 (3)	C23106/C23107	Apoptosis	2.1	1.2	0.9
	Interferon regulatory factor 4	B966	AU091159	Apoptosis	2	0.7	1.2
	Cathepsin B	B227	AU090814	Apoptosis	1.2	7.5	1.1
	Mitochondrial hinge protein	LA4 (10)	C23431	Apoptosis	ND	ND	3.2
	Cytochrome B	B296	AU090859	Apoptosis	1.2	1.4	2
	1,25 dihydroxyvitamin D3	WF5-21R	AU050667	Apoptosis	ND	ND	2
	Cytochrome C oxidase subunit-I	OL8	AU260722	Apoptosis	ND	0.2	ND
Cluster 5	Gelatinase-B	WB8 (7)	C82368	Metalloproteinase	2.6	5.5	1
(Inflammation genes)	Nephrosin	Z20	AU260535	Metalloproteinase	2.5	1.3	1.6
	protease component C3	WH12 (4)	C82226	Innate immunity	3.4	ND	ND
	Natural killer cell enhancing factor	B105	AU090730/AU090731	Innate immunity	0.4	1.7	0.8
	Sulfated glycoprotein	B942	AU091151	Inflammation	1.1	2.9	1.1
	Osteoclast stimulation factor	WF12 (3)	C82131/C82132	Inflammation	1.3	ND	2.4
	Homogentisate 1,2 dioxygenase	LH6 (9)	C23426	Inflammation	ND	ND	0.2

Table 2 *Continued*

Cluster No.	Gene name	Clone Identification	Gene accession No	Function	Expression levels Day 1†	Day 3‡	Day 7§
Cluster 6	ADP/ATP transaldorase 2	B235	AU090822	Metabolism	3.9	ND	1
(Other regulated genes)	Glucose-6-phosphatase	LA1 (9)	C23369	Metabolism	3.5	ND	1
	sodium/potassium transporting ATPase	N4	AU260498	Metabolism	2.9	1.3	1.6
	Transketolase	HC11 (1)	C23499	Metabolism	2.6	ND	1.1
	Myosin1-A	WE5-8R	AU050053	Protein metabolism	2.3	ND	1.4
	Hypothetical Protein DKDZp762H186.1	Y28	AU260766	Unknown	2.2	0.9	1.1
	Calreticulin	LD6 (10)	C23456	Molecular chaperone	2.1	0.9	1.1
	Protein arginine N-methyltransferase 3	WH9-20F	AU050616/AU050617	Methylation	1.5	4.9	1.1
	Ketohexokinase	L317	AU261082	Metabolism	1.3	4	1.5
	Profilin	B274	AU090848	Cell differentiation	0.9	3.4	1.4
	NADH dehydogenase subunit-5	OL34	BAA89043	phosphorylation	1	2.5	1.2
	NADH dehydogenase subunit-3	OL32	BAA89040	phosphorylation	1.6	2.3	1.6
	Growth harmone	OL23	BAA06159	Endocrine metabolism	1.2	2.1	1.2
	Syntaxin binding protein 3	L217	AU261026	Phosphorylation	1.5	2	1.3
	Neural SRC interating protein	Y147	AU260859	Phosphorylation	1.1	2	1.4
	Glycil tRNA ligase	B230	AU090816	Unknown	ND	ND	3.2
	Glycine decorboxylase	LB6 (5)	C23180	Metabolism	ND	ND	2.3
	Ribophorin	M90	AU260489	Protein metabolism	0.4	ND	0.4
	Histone H1	Y126	AU260840	DNA packing	0.4	ND	0.4
	CG8666 gene product	X9	AU260729	Unknown	ND	0.3	0.8
	Cystatin	LD8 (10)	C23459	Ca+2 binding	0.8	0.7	0.5
	Glucocorticoid receptor	OL19	O73673	Immunosupression	1.1	ND	0.5
	Calcium binding protein SDF4	WC3-23R	AU050772	Ca+2 binding	ND	ND	0.4
	Neutral calponin	WD1 (2)	C82034	Cell differentiation	1.2	ND	0.3
	Fibrinogen B-beta subunit	LC12 (6)	C23252/C23253	Blood clotting	ND	ND	0.3
Cluster 7	uk	O63	AU260562	Unknown	2.7	ND	0.9
(Unknown genes)	uk	WA6 (2)	C82006,C82007	Unknown	2.6	ND	ND
	uk	LB6 (10)	C23439/C23440	Unknown	2.3	ND	1.2
	uk	WH9 (3)	C82149/C82150	Unknown	2.3	ND	0.7
	uk	WH3-10F	AU050166	Unknown	2.3	0.8	1
	uk	L28	AU050541	Unknown	2.2	1.5	1.3
	uk	WC2-22R	AU050705	Unknown	2.1	ND	2.2
	uk	WF12-18F	AU050518	Unknown	2	ND	1.7

uk	WG5 (4)	C82212/C82213	Unknown	2	1.2	0.9
uk	WA4-12F	AU050212	Unknown	2	0.8	2.2
uk	B114	AU090735	Unknown	1.5	2	2.1
uk	WE8 (3)	C82115	Unknown	1.3	2.4	0.5
uk	WC12-12R	AU050230	Unknown	1.1	2.2	1
uk	WA11-10R	AU050134	Unknown	1	3.2	1.1
uk	WC2-9R	AU050090	Unknown	1	3.1	ND
uk	WF1-21F	AU050662	Unknown	ND	ND	3.7
uk	LB3 (8)	C23331	Unknown	0.4	ND	0.5
uk	HC6 (1)	C23496/C23497	Unknown	0.4	1.6	0.5
uk	B406	AU090904	Unknown	0.3	1.3	1
uk	B529	AU090990	Unknown	ND	0.3	ND
uk	L348	AU261109	Unknown	ND	0.3	ND
uk	L108	AU260964	Unknown	ND	0.3	1
uk	LB9 (8)	C23335	Unknown	ND	ND	0.5
uk	B148	AU090760	Unknown	ND	ND	0.5
uk	B1197	AU091263	Unknown	ND	ND	0.5
uk	LC6 (9)	C23384	Unknown	ND	ND	0.5
uk	LC6 (7)	C23300/C23301	Unknown	ND	ND	0.4
uk	B1156	AU091232	Unknown	ND	ND	0.4
uk	Z25	AU260880	Unknown	ND	ND	0.3
uk	WA6 (1)	C81936/C81937	Unknown	ND	ND	0.3
uk	B303	AU090862	Unknown	ND	ND	0.3

[†]Interleukin-(IL)1β – induced gene expression level after day 1.
[‡]IL-1β – induced gene expression level after day 3.
[§]IL-1β – induced gene expression level after day 7.
ND, not detected; uk, unknown.

findings of other research groups. The induction of chemokine receptor A in our study, indicates that IL-1β acts as a chemo attractant, as previously reported.[23]

IL-1β plays a central role in host defense. It induces differentiation and expression of surface immunoglobulin in pre B cells, proliferation of Th2 cells in combination with stimulation through the TCR, and proliferation and immunoglobulin secretion of mature B cells.[24] Our finding that JF-IL-1β induced the expressions of IgM, CD20, β-2-microglobulin and the immunoglobulin light chain (Table 2) suggests that it has a role in humoral immunity. CD20 also has a role in B cell activation and differentiation.[25] The increase in the expression of MHC class I antigens in our study raises the possibility that IL-1β acts as a mediator of cellular immunity. This is because the adjuvant effect of IL-1β requires increased cytotoxic lymphocyte activity.[26] CD3 gamma/delta plays a role in TCR complex assembly and in the signal transduction mechanism.[27] Surprisingly, CD3 gamma/delta was down-regulated in our experiment.

IL-1β in mammals is reported to be a signaling molecule for communication between the immune system and other internal systems.[28] Toll-like receptor 1 (TLR-1), SH3P2 and Pleckstrin 47 were induced in response to IL-1β in our study (Table 2). TLR-1 is expressed ubiquitously and at rather high levels. It associates with and regulates the TLR-2 response. TLR-2 activates the NF-kB pathway, which regulates cytokine expression through MyD88. Activation of the NF-kB pathway initiates the adaptive immune response by producing inflammatory cytokines such as IL-1, IL-6, IL-8 and IL-12.[29] SH3P2 protein (28 kDa) has a short proline-rich stretch and a SH3 domain at the amino terminus, followed by three ankyrin repeats. Proteins containing SH3 domains are essential in several well characterized signaling pathways, including the Ras/MAPK pathway, which is involved in cellular division, differentiation and cytoskeleton reorganization in response to growth factor receptor activation. Interaction of SH3P2 with Cb1-induced Src-mediated tyrosine phosphorylation of SH3P2.[30] Transcription factors that activate IL-1β gene expression include NF-kB, AP-1 and a tyrosine phosphorylated protein.[31] We believe that IL-1β-induced SH3P2 plays a role in tyrosine phosphorylation and MAPK signal transduction pathway. cAMP pathways are involved in regulating transcription of IL-1β.[32] cAMP-inducing agents and IL-1 itself have been shown to sustain mRNA levels for over 24 h in human blood monocytes.[33] In this study, as expected, IL-1β down-regulated the inducible cAMP early repressor (Table 2).

IL-1β performs a variety of roles, acting sometimes as a pro-apoptotis factor and sometimes as an anti-apoptosis factor. In the latter case, it protects against TNF-mediated cell death by arresting cell cycling.[34] IL-1 is processed and released during apoptosis.[35] Cytochrome c oxidase is a pro-apoptosis molecule. Several apoptotic pathways release cytochrome c from the mitochondrial intermembrane space, resulting in the activation of downstream caspases.[36] IRF4 activates the basal transcription of IL-1β promoter in macrophages and fibroblasts[37] and is essential for the function of B cells and cytotoxic T lymphocytes.[38] Cathepsin B has different roles. It protects cytotoxic lymphocytes from self-destruction after degranulation.[39] TRAF15 is an apoptosis-related protein according to entries in the databases. Cytochrome b is an essential component of the super oxide-generating oxidase.[40] Our results seem to show that IL-1β activates apoptosis molecules, both pro and anti.

Many other genes with different functions were also induced in response to IL-1β, such as matrix metalloproteinases (MMP; e.g. gelatinase) and oxidative phosphorylation-related NADH dehydrogenases. MMP genes are strongly induced by IL-1β.[7] IL-1 has been shown to up-regulate monocyte and macrophage MMP (gelatinase) production *in vitro*.[41] As expected, the glucocorticoid receptor was down-regulated in our study (Table 2). Corticosteroids inhibit the transcription of IL-1. Increased synthesis of I kB with decreased translocation of NF-kB is thought to account for the suppressive effect of glucocorticoids on cytokine synthesis.[42] Some unknown genes were regulated up or down in our experiment. In our previous EST analyses, 35–40% of the genes were unknown genes which did not show any homology to the known genes in the gene bank databases. Some of these genes are expected to have immune function. The nucleotide sequencing and protein expression patterns are expected to give more complete information about these genes.

In conclusion, our results demonstrate that the immune response in Japanese flounder is significantly influenced by IL-1β, both *in vitro* and *in vivo*. The fish injected with IL-1β cDNA using a DNA-injection method stimulated the expression of immune-related genes, suggesting that Japanese flounder IL-1β gene has functional similarity with that of mammalian IL-1β. Dissociation between transcription and translation is characteristic of IL-1β.[43] The microarray analysis made it possible to identify these immune responses at the transcriptional level. Further studies are needed to understand the changes at the translational level.

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