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NOTE

Microarray analyses of gene expression in Japanese flounder *Paralichthys olivaceus* leucocytes during monogenean parasite *Neoheterobothrium hirame* infection

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ABSTRACT: In this study, the gene expression patterns of peripheral blood leucocytes (PBL) from Japanese flounder *Paralichthys olivaceus* were analyzed during the course of monogenean parasite *Neoheterobothrium hirame* infection in order to select candidates for molecular biomarkers of infection. cDNA microarray analysis was performed to compare the gene expression patterns of PBL between infected and non-infected fishes. Among the 797 genes analyzed, 45 genes (5.6%) changed their expression levels. These genes included specific and non-specific immune-related genes (matrix metalloproteinase[MMP]-9, MMP-13, leukotriene B4 receptor, CD20 receptor, MHC [major histocompatibility complex] Class I, MHC Class II β-chain, immunoglobulin light chain and immunoglobulin heavy chain). Significant up- and down-regulation of some unknown genes was also observed. Several candidates for infection-marker genes were selected for further study. These genes included MMP-9, MMP-13, leukotriene b4 receptor, CD20 receptor, immunoglobulin heavy chain, immunoglobulin light chain and unknown genes coded as B613, E25, LB3(8), WE2(3), WE8-18R and WF12-18R.

KEY WORDS: Japanese flounder · *Neoheterobothrium hirame* · Microarray analysis · Gene expression profiling · Leucocyte

INTRODUCTION

Early identification of a pathogen is important for the prevention of the progression and outbreak of disease. In the field of medical science, the composition of peripheral blood leucocytes (PBL) has been utilized as a source of information with which to monitor the condition of the host because it reflects the host's physiological state. PBL classification and counting is an important diagnostic technique in the detection of many illnesses. Furthermore, in teleost hosts, the composition and activity of PBL changes in response to inflammation. Hence, monitoring the physiological state of PBL may provide information about the health condition of the host. Cell regulation essentially occurs via changes in RNA levels. The genes specifically expressed under certain pathogen infections may be utilized as infection-markers for early diagnosis of diseases.

DNA microarray is a powerful tool with which to track global changes in gene expression under different physiological conditions. It has been utilized to monitor host responses to viral haemorrhagic septicemia virus (VHSV) (Byon et al. 2006) and *Edward-*

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siella tarda infection (Matsuyama et al. 2006) in Japanese flounder *Paralichthys olivaceus*. In this study, we chose *Neoheterobothrium hirame* as an example of a monogenean infection in order to select the genes that could be utilized as infection-markers.

Neoheterobothrium hirame infection has been reported in both cultured and wild Japanese flounder (Ogawa 1999). High mortality was observed among flounders that were experimentally challenged with the parasite (Yoshinaga et al. 2001). Mature *N. hirame* infests the buccal cavity wall by embedding its posterior deep into the host tissue and sucking blood through the anterior, causing anemia in the host (Anshary & Ogawa 2001). The parasite causes necrosis and intensive infiltration of inflammatory cells in tissues around the attachment sites. Nakayasu et al. (2005) suggested that monocytes/macrophages, granulocytes and cells with large, electron-dense granules may possibly be involved in the elimination of the parasite by cellular response, because these cells infiltrated and directly adhered to the parasites, and destroyed the integument and inner tissue of the parasites. There is no doubt about the involvement of leucocytes in the host response, but their role in the defense activity against *N. hirame* is unclear. In this study, to elucidate the transcriptional response of leucocytes to *N. hirame* infection and to identify candidates for molecular biomarkers of infection, we analyzed the gene expression patterns of PBL in response to *N. hirame* infection using cDNA microarray.

MATERIALS AND METHODS

Fish and *Neoheterobothrium hirame* infection. Japanese flounder (average body weight: 417 g) were maintained at 25°C in flow-through 120 l tanks. The flounder were infected with *N. hirame* as described by Yoshinaga et al. (2001). At 2, 5, 10 and 21 d after the first visual observation of adult worms in the buccal cavity wall, 4 fishes were sampled randomly for microarray analysis. Six healthy fishes were used as controls. Hemoglobin concentration (Hb) of each individual was determined by the cyanomethemoglobin method. Hb was analyzed by Student's *t*-test and significant differences were determined at $p < 0.05$.

Microarray analysis. PBL was isolated from peripheral blood using Histopaque (density: 1.077 g ml^{-1}) (Sigma), and total RNA was extracted using TRIzol (Invitrogen). To minimize the individual gene expression variation that may arise among samples, RNA samples within each group were pooled.

We used a Japanese flounder *Paralichthys olivaceus* cDNA microarray, which contained 797 clones that were obtained from the EST (expression sequence tag) library (Kurobe et al. 2005). Each gene was spotted in duplicate to facilitate comparison during the analysis. cDNA was synthesized from 25 µg of total RNA using a Labelstar array kit (Qiagen). cDNA synthesized from *Neohetero bothrium hirame*-challenged fish samples was labeled with Cy5 (carboxymethyl indocyanine-5), while the control samples were labeled with Cy3. An equal volume of Cy3- or Cy5-labeled first strand cDNA was used for microarray analysis. Normalization of signal intensity was performed using the elongation factor 1 α gene.

The signal intensity was calculated as the mean intensity of duplicate spots minus the background signal. Signals of clones whose intensity was below 1000 were removed. The ratio was calculated as the infected sample signal intensity divided by the control signal intensity. Genes with ratios over 2.0 were considered to be up-regulated, and genes with ratios less than 0.5 were considered to be down-regulated. The gene expression data from selected genes were hierarchically clustered using the Cluster program (Eisen et al. 1998).

RESULTS AND DISCUSSION

Because of the difficulty in controlling infection of parasites in laboratory studies, host response against parasite infection in fish has received little attention. *Neoheterobothrium hirame* is a good model for the elucidation of parasite-host interactions because its ecology has been thoroughly studied and the method for artificial infection is well established (Yoshinaga et al. 2001). In this study, infection was experimentally

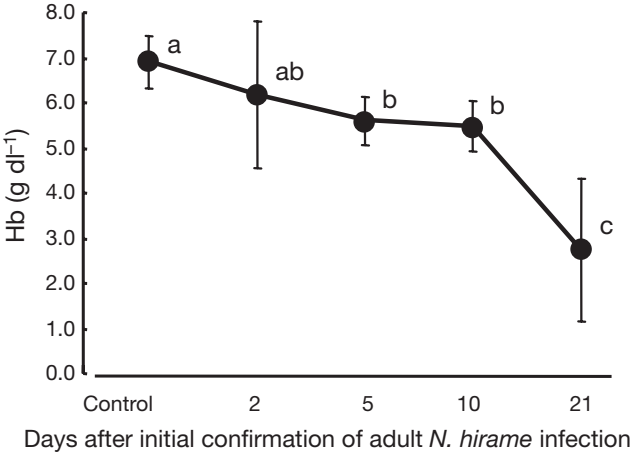

Fig. 1. *Paralichthys olivaceus*. Changes in the concentration of Hb in peripheral blood of Japanese flounder challenged with *Neoheterobothrium hirame* (mean ± SD). Letters (a,b,c) indicate significant differences at $p < 0.05$

reproduced. Hb continuously decreased with the infection of the parasites in the buccal cavity wall, and was significantly lower at 5, 10 and 21 d compared with the control (Fig. 1).

Among the 797 genes analyzed, 45 (5.6%) changed their expression levels upon infection with *Neoheterobothrium hirame.* Genes for which the expression level was altered by *N. hirame* infection were categorized

Table1. *Paralichthys olivaceus*. Gene expression profile of PBL at 2, 5, 10 and 21 d after initial confirmation of adult *Neoheterobothrium hirame* infection. Values indicate ratios compared with the control. Signals of clones of intensity <1000 indicated by ND. Ratios of >2.0 (in **bold**) and <0.5 (underlined) indicate up- and down-regulation, respectively

Gene name	Function	GenBank accession no.	Relative expression Day 2	Day 5	Day 10	Day 21
Cluster 1						
Hormone-sensitive lipase	Lipase	AU090735	**2.0**	**2.2**	1.2	0.9
MMP-9	Protease	AU050212	**2.4**	**2.4**	1.6	0.6
MMP-13	Protease	C82102	**2.4**	1.9	1.8	1.2
CD20 receptor	B cell differentiation	C82121	**2.2**	1.9	**2.2**	1.0
Zinc finger protein ZNF183	Transcription factor	AU050398	1.9	1.9	**2.1**	1.2
Immunoglobulin heavy chain	Humoral defense	AU260744	0.8	1.1	**2.8**	**2.0**
Immunoglobulin light chain	Humoral defense	AU260797	1.5	1.1	**3.1**	0.6
Leukotriene b4 receptor	Chemotaxis	AU091248	**2.1**	1.5	ND	ND
Tyrosine kinase	Kinase	AU050573	1.3	ND	**2.3**	0.9
Invariant chain-like protein 2	Unknown	AU091270	1.1	1.2	**2.1**	0.8
Actin beta	Actin	AU090737	1.9	1.8	**2.0**	1.3
Unknown		C82107/C82108	**2.3**	1.8	**2.6**	1.5
Unknown		AU261005	1.2	1.2	**2.2**	ND
Unknown		AU260854	ND	1.2	**2.5**	ND
Unknown		AU090736	ND	1.4	**8.5**	0.6
Unknown		AU260789	ND	1.3	**3.4**	0.8
Unknown		c23003	ND	1.2	**2.4**	ND
Unknown		AU050518	1.7	1.8	ND	0.4
Cluster 2						
MHC Class I	Antigen presentation	AU260510	0.8	1.2	**2.1**	ND
MHC Class II β-chain	Antigen presentation	AU090795	1.3	1.7	**2.4**	0.9
Cytochrome B	Metabolism	BAA19157	1.3	1.5	**2.7**	1.0
Cytochrome C oxidase subunit1	Metabolism	AU090894	1.9	1.9	**4.8**	1.4
Cytochromee oxidase subunint I	Metabolism	AU090796	ND	1.6	**3.7**	1.5
Unknown		C82315	1.2	1.6	**3.0**	ND
Unknown		AU050145	1.4	1.7	**2.6**	0.9
Unknown		AU091252	1.3	**2.2**	**3.9**	1.1
Unknown		AU091089/AU091090	ND	1.7	**2.3**	1.1
Unknown		AU090817	1.5	1.8	**3.5**	0.9
Unknown		AU090855	1.0	ND	**2.3**	0.9
Unknown		AU091098	1.3	1.5	**3.6**	0.9
Unknown		AU050512	1.3	1.7	**3.3**	ND
Unknown		AU261058	ND	1.2	**2.2**	0.8
Unknown		AU261076	1.3	1.5	**3.4**	0.9
Unknown		C23331	1.0	1.5	**4.5**	0.5
Unknown		AU260509	0.7	1.2	**2.0**	0.7
Unknown		AU050475	0.9	1.2	**2.4**	0.6
Cluster 3						
Thrombospondin	Cell migration and proliferation	C82377	ND	0.7	ND	0.4
Equistatin precursor	Protease inhibitor	AU260462	1.0	0.8	1.5	0.5
Unknown		AU260408	0.7	0.3	**3.1**	0.5
Unknown		c23260/c23261	0.9	1.2	1.6	0.5
Unknown		c23206/c23207	1.1	0.8	1.2	0.4
Cluster 4						
Unknown		AU260405	0.5	0.4	ND	ND
Unknown		AU090918	ND	0.5	ND	0.4
Unknown		AU091002	0.5	0.8	1.7	ND
Unknown		AU091060	0.8	1.2	1.4	0.5

into 4 clusters (Table 1). Clusters 1 and 2 consisted of genes that were up-regulated by *N. hirame* infection, while Clusters 3 and 4 consisted of genes that were down-regulated by *N. hirame* infection. Specific and non-specific immune-related genes were included in Clusters 1 or 2 (matrix metalloproteinase[MMP]-9, MMP-13, leukotriene B4 receptor, CD20 receptor, MHC Class I, MHC Class II β-chain, immunoglobulin light chain and immunoglobulin heavy chain). While Hb did not decrease and no remarkable symptoms were observed 2 d after infection with adult worms was confirmed, 8 genes altered their expression level at this sampling point. These genes may be useful markers with which to detect the onset of infection.

Expression of some of the immune-related genes was not affected by *Neoheterobothrium hirame* infection (data not shown). These genes included CXC chemokine, tumor necrosis Factor α (TNFα), cathepsin B and lymphocyte antigen LY-6G.1 precursor. Although CXC chemokine and TNFα are important elements with which to induce inflammatory responses, expression of these 2 cytokines was not altered by *N. hirame* infection. Their expression might be regulated at the site of inflammation, even though they are not regulated in the peripheral blood.

In order to explore the host-parasite interaction, it is interesting to observe the events at the site of infection as they progress. Adult *Neoheterobothrium hirame* infect the skin of the buccal cavity wall. The skin is an important site at which to prevent invasion of pathogens. Important cellular components of the skin for immune response are mucosal cells and leucocytes. These cells communicate to eliminate the parasites through various cytokines, which results in the production and release of hostile molecules (Buchmann 1999). As reported by Nakayasu et al. (2005), *N. hirame* induces inflammatory reactions at the infected site. Further study focusing on the reaction at the site of infection should be conducted to improve our understanding of the host-parasite interaction.

Comparing with the previous study that analyzed the gene expression of PBL after infection with *Edwardsiella tarda* (Matsuyama et al. 2006), some genes were commonly up-regulated with the infection of *Neoheterobothrium hirame* and *E. tarda*. These genes included MMP-9, MMP-13, CD20 receptor and unknown genes coded as LB3(8) and WE8-18R. In contrast, both of the pathogens down-regulated the expression of the equistatin precursor gene. These genes have great potential as markers of pathological condition because they were commonly regulated in response to 2 extremely different infectious agents. *N. hirame* is a parasite that infests the buccal cavity wall, whereas *E. tarda* is a bacterium that causes systemic infection. MMPs are a family of zinc metallo-endopeptidases that regulate cell matrix composition (Johnson et al. 1998). They have also been evaluated as markers of inflammation, including as markers of auto-immune diseases and cancer (Johnson et al. 1998). MMP-9 is important for leukocyte migration and inflammation owing to its ability to degrade basement membranes and components of the extra cellular matrix (Dubois et al. 1999). Induction of MMP-9 in mice during nematoda parasite infection has also been reported (Lai et al. 2005). MMP-13 has a key role in the MMP activation cascade (Leeman et al. 2002). It also contributes to wound repair (Ye et al. 2000).

The genes that were regulated differently in response to each pathogen are potential markers of infective agents. Such genes identified in this study were the immunoglobulin heavy chain, immunoglobulin light chain and unknown genes coded as WF12-18R and B613. Immunoglobulin heavy chain and light chain coding genes were up-regulated upon *Neoheterobothrium hirame* infection (this study), but were down-regulated upon *Edwardsiella tarda* infection (Matsuyama et al. 2006). In contrast, unknown genes WF12-18R and B613 were down-regulated upon *N. hirame* infection, but were up-regulated upon *E. tarda* infection. Recent studies demonstrated that leucocytes recognize pathogens differently through toll-like receptors, inducing the overlap or unique host response to individual pathogens (Akira et al. 2006). This system is conserved among many vertebrates including teleosts. Japanese flounder may differentiate between *N. hirame* and *E. tarda* to induce different host responses.

Elevation of the specific serum antibody titer against *Neoheterobothrium hirame* after adult parasite infection was demonstrated using ELISA techniques (Tsutsumi et al. 2003). Additionally, up-regulation of humoral immunity related genes was observed in this study. These genes included immunoglobulin heavy chain, immunoglobulin light chain, MHC Class II β-chain and the CD20 receptor. The CD20 receptor gene encodes a B-lymphocyte surface molecule, which plays an important role in B-cell activation, proliferation and differentiation (Tedder & Engel 1994). *N. hirame* infection seems to induce the humoral immunity.

In this study, we focused on the gene expression of PBL in order to search for molecular biomarkers of infection. PBL are comprised of several cell subsets. Although data from mixed cell populations are difficult to interpret, mixed cell populations represent a comprehensive movement of the host response. We selected some genes as candidates for infection markers. Concurrently, this study elucidated part of the host response against parasite infection, including induction of the humoral immune response. Further analysis of gene expression patterns targeted at

specific cell subsets is necessary if we are to improve our understanding of detailed host anti-parasite responses.

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