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RESEARCH REPORT

Vertebrate interleukins originated in invertebrates?

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Abstract

Previous studies have demonstrated that invertebrate immune and neural tissues contain mammalian-like cytokines, which activate specific cellular functions. Therefore, it was of interest to attempt to identify these molecules via Applied Biosystems Human Genome Survey Arrays. The array was used to analyze the transcriptional profiles of *Mytilus edulis* RNA samples. The Applied Biosystems Human Genome Survey Array contains 31,700 60-mer oligonucleotides probes representing a set of 27, 868 individual human genes and more than 1,000 control probes. We show interleukin-like and tumor necrosis factor-like genes among other cytokine-like genes significantly expressed in this invertebrate tissue with a signal to noise value greater than 2. In morphine treated tissue additional cytokine genes were expressed. These cytokine-like genes are directly related to previously discovered molecules in invertebrates, suggesting that they first appeared earlier in evolution.

Key words: mussel; *Mytilus edulis*; cytokines; microarray

Introduction

Mytilus edulis neural tissues contain both immune- and neural-like signaling molecules found in mammals (see Stefano, 1982, 1990a, 1992). In regard to catecholamines, the neural tissues of *Mytilus* contain both dopamine and norepinephrine (Stefano, 1982, 1990b). In reference to cytokine-like molecules, interleukin (IL)-1-, IL-6- and tumor necrosis factor (TNF)-like molecules have been identified via radio-immune assay in *Mytilus* ganglia and immune tissues (Hughes *et al.*, 1990, 1991a; Hughes and Chin, 1994; Scharrer *et al.*, 1996). Thus, invertebrate ganglia and immune tissues appear to have the potential to respond like mammalian tissues to these signaling molecules. Concerning the signaling of these molecules, invertebrate immune tissues, i.e., immunocytes, respond to IL-1 and IL-6 by undergoing conformational changes indicative of becoming activated, i.e., amoeboid, including stimulating mobility (Hughes *et al.*, 1990, 1991a,b; Hughes and Chin, 1994; Scharrer *et al.*, 1996).

Thus, given their presence and action in invertebrate physiological systems it was of great interest to determine if human microarray chips would also show that they are present given the many biochemical and pharmacological similarities.

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Materials and Methods

Mytilus edulis were harvested from the shores of Long Island Sound at Mattituck, New York during the month of March. Animals were then transported to the laboratory in chilled seawater (4-10 °C). In the laboratory, they were maintained as previously described in detail (Stefano *et al.*, 1994). *M. edulis* pedal ganglia (20 per array) were dissected and kept on ice until needed. In order to determine the presence of

Table 1 Interleukin-like, tumor necrosis factor-like, and other cytokine-like genes that were significantly expressed as analyzed by the Human Genome Survey Microarray (Applied Biosystems) with a signal to noise value greater than 2 in the untreated *Mytilus edulis* pedal ganglia tissue.

Tumor necrosis factor (TNF)-like molecules present	
TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a, activator of NFKB
TNFRSF25; KIAA0720	Tumor necrosis factor receptor superfamily, member 25; putative NFKB activating protein
Interleukins-like molecules present	
IL16	Interleukin 16 (lymphocyte chemoattractant factor)
IL7	Interleukin 7
IL31RA	Interleukin 31 receptor A
IL15	Interleukin 15
IL11RA	Interleukin 11 receptor, alpha
IL18BP	Interleukin 18 binding protein
IL23R	Interleukin-23 receptor
Additional cytokine-like molecules present	
Chemokine and chemokine receptor activity	
CCL23	Chemokine (C-C motif) ligand 23
CCRL2	Chemokine (C-C motif) receptor-like 2
CCL1	Chemokine (C-C motif) ligand 1
CCR9	Chemokine (C-C motif) receptor 9
CXCR3	Chemokine (C-X-C motif) receptor 3
CCL13	Chemokine (C-C motif) ligand 13
CCL15; CCL14	Chemokine (C-C motif) ligand 15; chemokine (C-C motif) ligand 14
CCL19	Chemokine (C-C motif) ligand 19
CCL24	Chemokine (C-C motif) ligand 24
MGC12815; CCL3L1; CCL3	Chemokine (C-C motif) ligand 3-like, centromeric; Chemokine (C-C motif) ligand 3-like 1; Chemokine (C-C motif) ligand 3
CCR6	Chemokine (C-C motif) receptor 6
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
Cell growth and growth factor activity	
GDF8	Growth differentiation factor 8
GDF2	Growth differentiation factor 2
EBAF	Endometrial bleeding associated factor (left-right determination, factor A; transforming growth factor beta superfamily)
BMP1	Bone morphogenetic protein 1
MYH11	Myosin, heavy polypeptide 11, smooth muscle
Cytokinesis and other cell-cycle activity	
CDK6	Cyclin-dependent kinase 6
CDC23	CDC23 (cell division cycle 23, yeast, homolog)
CCND3	Cyclin D3
CDC25A	Cell division cycle 25A
CCNC	Cyclin C
CCNL1	Cyclin L1
CDC14A	CDC14 cell division cycle 14 homolog A (*S. cerevisiae*)
PARD6A	Par-6 partitioning defective 6 homolog alpha (*C.elegans*)
PARD3	Par-3 partitioning defective 3 homolog (*C. elegans*)
PRC1	Protein regulator of cytokinesis 1
STAT1	Signal transducer and activator of transcription 1, 91kDa
NEDD5	Neural precursor cell expressed, developmentally down-regulated 5
3-Sep	Septin 3
DOCK1	Dedicator of cytokinesis 1
Immune activity	
LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
OSM	Oncostatin M
PF4	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)

TLR2	Toll-like receptor 2
IFNK	Interferon, kappa
Other	
DAPK1	Death-associated protein kinase 1
LATS1	LATS, large tumor suppressor, homolog 1 (*Drosophila*)
PIN1	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
PIN1L	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1-like
MOBK1B	MOB1, Mps One Binder kinase activator-like 1B (yeast)
SDFR1	Stromal cell derived factor receptor 1
C17	Cytokine-like protein C17
EPOR	Erythropoietin receptor
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
ACVR2B	Activin A receptor, type IIB
NRP1	Neuropilin 1
PBEF1	Pre-B-cell colony enhancing factor 1
OBRGRP; LEPR	Leptin receptor gene-related protein; Leptin receptor
TLT4	TREM-like transcript 4
LOC392255	Similar to growth differentiation factor 16

aforementioned molecules upon stimulation with a neuroimmune effecter using microarray, ganglia were incubated at 4 °C in filtered seawater or treated with 1 µM morphine for 18 h.

Applied Biosystems expression array analysis

Applied Biosystems Human Genome Survey Arrays were used to analyze the transcriptional profiles of RNA samples. The Applied Biosystems Human Genome Survey Array contains 31,700 60-mer oligonucleotides probes representing a set of 27, 868 individual human genes and more than 1,000 control probes. Sequences used for microarray probe design are from curated transcripts from the Celera Genomics Human Genome Database (www.celeradiscoverysystem.com), RefSeq transcripts that have been structurally curated from the LocusLink (http://ncbi.nlm.nih.bov/LocusLink/refseq.html) public database, high-quality cDNA sequences from the Mammalian Gene Collection (MGC) (http://mgc.nci.nih.gov) and transcripts that were experimentally validated at Applied Biosystems.

Total RNA from 20 *M. edulis* pedal ganglia was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The tissue was lysed in 600 µl buffer RLT and homogenized by passing the lysate 5 times through a 20-gauge needle fitted to a 3 ml syringe. The samples were then processed following the manufacturer's detailed instructions. In the final step, the RNA was eluted with 50 µl of RNase-free water by centrifugation for 1 min at 10,000 rpm. Quality of the RNA was analyzed using Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) using the total RNA nanochip according to manufacturer's protocol. Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 µg of total RNA using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit v 2.0 and manufacturer's protocol. Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following manufacturer's protocol. To each chip, 15 µg of labeled cRNA targets were hybridized at 55 °C for 19 h.

AB1700 Expression System software was used to extract assay signal, and assay signal to noise ratio values from the microarray images. To select expressed genes, the gene list was further filtered by removing genes with a signal to noise value less than two.

Results

The previously discovered invertebrate cytokine-like molecules include tumor necrosis factor-like molecules as well as IL-1-, IL-2-, IL-4-, IL-6- and IL-10-like molecules. Table 1 demonstrates interleukin-like and tumor necrosis factor-like genes among other cytokine-like genes that were significantly expressed as analyzed by the Human Gene Survey microarray (Applied Biosystems) with a signal to noise value greater than 2 in the untreated *M. edulis* pedal ganglia tissue. With a signal to noise value greater than 2, all genes expressed are thus considered to have a strong significant presence. Tumor necrosis-like factors were present in the untreated tissue. Additionally, several interleukin-like molecules also were present.

In the morphine treated tissue, however, several additional genes were expressed (Table 2). Among these genes expressed was IL-10, an interleukin-like molecule previously demonstrated in

Table 2 Interleukin-like, tumor necrosis factor-like, and other cytokine-like genes that were significantly expressed in *Mytilus edulis* pedal ganglia after morphine treatment. Genes with a signal to noise ratio greater than 2 as analyzed by the Human Genome Survey Microarray (Applied Biosystems) were listed.

Tumor necrosis factor (TNF)-like molecules present	
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a
TNFSF12-TNFSF13; TNFSF12; TNFSF13	Tumor necrosis factor (ligand) superfamily, member 12-member 13; tumor necrosis factor (ligand) superfamily, member 12; tumor necrosis factor (ligand) superfamily, member 13
Interleukin-like molecules present	
IL17RB	Interleukin 17 receptor B
IL10	Interleukin 10
IL23A	Interleukin 23, alpha subunit p19
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)
IL31RA	Interleukin 31 receptor A
IL18	Interleukin 18 (interferon-gamma-inducing factor)
IL15RA	Interleukin 15 receptor, alpha
Additional cytokine-like molecules present	
Cytokinesis and other cell-cycle activity	
CDC25B	Cell division cycle 25B
ANAPC5	Anaphase promoting complex subunit 5
CDK7	Cyclin-dependent kinase 7 (MO15 homolog, Xenopus laevis, cdk-activating kinase)
CDK4	Cyclin-dependent kinase 4
ROPN1	Ropporin, rhophilin associated protein 1
SOCS2	Suppressor of cytokine signaling 2
PNUTL1; GP1BB	Peanut-like 1 (Drosophila); glycoprotein Ib (platelet), beta polypeptide
SPAG5	Sperm associated antigen 5
ANAPC4	Anaphase promoting complex subunit 4
CDC6	CDC6 cell division cycle 6 homolog (S. cerevisiae)
UBE2C	Ubiquitin-conjugating enzyme E2C
DOCK3	Dedicator of cytokinesis 3
Other	
CNTFR	Ciliary neurotrophic factor receptor
ASB9	Ankyrin repeat and SOCS box-containing 9
PREI3	Preimplantation protein 3
STAT2	Signal transducer and activator of transcription 2, 113kDa
GAB3	GRB2-associated binding protein 3
OBRGRP; LEPR	Leptin receptor gene-related protein; leptin receptor
ASB10	Ankyrin repeat and SOCS box-containing 10
CRLF3	Cytokine receptor-like factor 3
11-Sep	Septin 11
ASB1	Ankyrin repeat and SOCS box-containing 1
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta

Table 3 Additional interleukin-like genes expressed in presence of morphine as analyzed by the Human Genome Survey Microarray (Applied Biosystems) with a signal to noise ratio between 1 and 2.

Gene Name		Gene Ontology
IL1F10	Interleukin 1 family member 10 (theta)	Immune response, interleukin-1 receptor antagonist activity, extracellular space
IL1F5	Interleukin 1 family member 5, delta	Immune response; interleukin-1 receptor antagonist activity; extracellular space
IL1RL2	Interleukin 1 receptor-like 2	Interleukin-1, Type I, activating receptor activity, transmembrane receptor activity, integral to membrane
IL1RL1	Interleukin 1 receptor-like 1	Signal transduction, transmembrane receptor activity; receptor signaling protein activity;interleukin-1 receptor activity
IL1F9	Interleukin 1 family member 9	Cell-cell signaling; immune response; response to pest/pathogen/parasite; interleukin-1 receptor antagonist activity; extracellular space
IL6ST	Interleukin 6 signal transducer (gp 130, oncostatin M receptor)	Extracellular space; integral to membrane; protein binding; glycogen metabolic process; positive regulation of cell proliferation; regulation of Notch signaling pathway; signal transduction
IL4	Interleukin 4	Cholesterol metabolism; regulation of isotype switching; cell proliferation; B-cell differentiation; cellular defense response; T-helper 2 type immune response; connective tissue growth factor biosynthesis; chemotaxis, interleukin-4 receptor binding; extracellular space

invertebrates (Stefano *et al.*, 1999) and TNF-like molecules different from those expressed in the untreated tissue. Proinflammatory cytokines and TNF play a major role in inflammation response. The immunosuppressive effect of morphine treatment is demonstrated by a significant presence in expression of the anti-inflammatory IL-10-like molecule. Additionally, the significant presence of TNF receptor-like molecules indicates the down regulation of proinflammatory TNF-like molecules. The additional newly discovered cytokine-like molecules detected by microarray in both the untreated and morphine treated tissue provide researchers with a multitude of possible subjects for future investigation.

Given the logarithmic analysis supplied by the SpotFire for functional genomics program (SpotFire, Somerville, Maine), any positive signal to noise value indicates gene presence is in greater amounts than background noise. Furthermore, the gene sequence of the human transcript on the microarray chip is not identical to the gene sequence of the corresponding *M. edulis* transcript. However, the array hybridization, as well as the washes, used the same stringency as human nucleic acid assays and we were still able to detect approximately 5000 genes. It is thus important to note the presence of any additional neuro-immune significant interleukin-like signal molecules that were detected upon morphine treatment of *M. edulis* pedal ganglia tissue (Table 3). These interleukin-like genes are directly related to previously discovered interleukin-like molecules in invertebrates.

Discussion

As noted earlier, invertebrate ganglia, immunocytes, and microglia contain IL-1- and IL-6-like signaling molecules (Beck and Habicht, 1986; Hughes *et al*, 1990, 1991a; Paemen *et al*, 1992; Stefano, 1992; Stefano *et al*, 1992; Hughes and Chin, 1994; Scharrer *et al*, 1996). Based on these findings, one can surmise that an interleukin-like molecule secreted from these invertebrate cells may have the ability to release dopamine from neurons. Recently, Sawada and colleagues, as well as others, demonstrated that mammalian IL-1 and IL-2 and -4 have the ability to alter invertebrate neural ion channels in a stereoselective manner, further strengthening the hypothesis that these immunocyte-derived molecules can alter neural activities as well as stimulate them (Sawada *et al.*, 1991; Szucs *et al.*, 1992; Franchini *et al.*, 1996; Rozsa *et al.*, 1997; Kletsas *et al.*, 1998).

In previous and current research, measures are taken to confirm gene expression including TaqMan Probes (Applied Biosystems) and molecular methods including Western blotting. The ability of microarray to corroborate with and/or confirm an expanse of previous research is demonstrated in this study of cytokine-like molecules found in *M. edulis*. Given the comprehensive nature of a single microarray chip and the accuracy and precision of the data expressed by these chips, this research indicates that the use of microarray could be independently sufficient for determining gene expression.

In summary, it appears that immune-neural communication does occur in invertebrate neural tissues. Certainly, the opposite has also been shown, i.e., that neuropeptides can alter and direct invertebrate immune actions (see Stefano *et al.*, 1996). This research has been able to confirm such previous findings using microarray technology.

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