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Differential responses of normal human coronary artery endothelial cells against multiple cytokines comparatively assessed by gene expression profiles

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Abstract **Endothelial cells play an important role in terms of biological functions by responding to a variety of stimuli in the blood. However, little is known about the molecular mechanism involved in rendering the variety in the cellular response. To investigate the variety of the cellular responses against exogenous stimuli at the gene expression level, we attempted to describe the cellular responses with comprehensive gene expression profiles, dissect them into multiple response patterns, and characterize the response patterns according to the information accumulated so far on the genes included in the patterns. We comparatively analyzed in parallel the gene expression profiles obtained with DNA microarrays from normal human coronary artery endothelial cells (HCAECs) stimulated with multiple cytokines, interleukin-1β, tumor necrosis factor-α, interferon-β, interferon-γ, and oncostatin M, which are profoundly involved in various functional responses of endothelial cells. These analyses revealed that the cellular responses of HCAECs against these cytokines included at least 15 response patterns specific to a single cytokine or common to multiple cytokines. Moreover, we statistically extracted genes contained within the individual response patterns and characterized the response patterns with the genes referring to the previously accumulated findings including the biological process defined by the Gene Ontology Consortium (GO). Out of the 15 response patterns in which at least one gene was successfully extracted through the statistical approach, 11 response patterns were differentially characterized by representing the number of genes contained in individual criteria of the biological process in the GO only. The approach to dissect cellular responses into response patterns and to characterize the pattern at the gene expression level may contribute to the gaining of insight for untangling the diversity of cellular functions.**

Keywords: Cellular response; Endothelial cell; Cytokine; Gene expression; DNA microarray

1. Introduction

Endothelial cells that line the blood vessels are directly exposed to a variety of stimulatory factors in the blood. In the presence of the stimuli, endothelial cells appropriately modulate their responses with respect to the adhesion of leukocytes, vasorelaxation, and immunity and play an important role in maintaining homeostasis [1]. In addition, endothelial cells are also involved in pathological processes such as thrombosis, requisite neovascularization of solid tumors, and atherosclerosis [2–4]. Thus, it is important to understand in detail the responses of endothelial cells to a variety of exogenous factors.

Cytokines are factors that induce a wide range of responses from endothelial cells. It has been difficult to uncover the differences and similarities in the responses of endothelial cells to cytokines comprehensively. The reason is because several cytokines share common signaling pathways consisting of identical receptor subunits and exhibit similar responses to the expression of a small number of genes and to specific cellular phenotypes.

Thus, it should be efficient to comprehensively analyze in parallel multiple cellular responses against exogenous stimuli via an identical platform in order to reveal the molecular mechanism through which the heterogeneity of responses of endothelial cells is rendered against the exogenous factors represented by cytokines. One of the platforms to conduct these analyses in parallel includes comprehensive gene expression profiling with DNA microarrays. So far, several studies have been published on comprehensive analyses for responses of endothelial cells exposed to cytokines [5–7]. These studies consist of identifying genes the expression levels of which altered in an identical or different fashion; such identification is done by comparing the expression profiles obtained from endothelial cells exposed to two or three species of cytokines side by side comparing two samples on an identical microarray directly. Nevertheless, these direct comparisons among two or three species of cytokines should be insufficient to fully understand the differences and similarities in the heterogeneity of cellular responses to cytokines.

Therefore, we sought to minutely dissect the responses of endothelial cells to cytokines with comprehensive gene

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Abbreviations: IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-β, interferon-β; IFN-γ, interferon-γ; OSM, oncostatin M

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expression profiles obtained from normal human coronary artery endothelial cells (HCAECs) that were exposed to the cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-β (IFN-β), interferon-γ (IFN-γ), oncostatin M (OSM), which are involved in a variety of cellular functions and partially share signal transduction pathways [8–10]. We obtained the gene expression profiles with synthetic DNA microarrays containing approximately 22000 species of probes and analyzed them in parallel. Here, we demonstrate that simultaneous analyses of the expression profiles obtained with these five cytokines enabled us to identify genes the expression levels of which altered in single and multiple cytokines-specific manners and to minutely dissect the cellular responses of HCAECs to the cytokines.

2. Materials and methods

2.1. Cells

HCAECs (Product code, CC-2585; Age, 32Y) were purchased from Cambrex Corp. (NJ, USA) and cultured under 5% CO_2 at 37 °C with EGM-2-MV medium (Cambrex Corp.) containing 5% fetal bovine serum (FBS), hydrocortisone, human fibroblast growth factor (hFGF)-B, vascular endothelial growth factor (VEGF), human recombinant insulin-like growth factor (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), and gentamicin/amphotericin B according to the manufacturer's instructions. HCAECs were split at 1:4 with 0.02% EDTA and 0.25% trypsin at a confluent density.

2.2. Cytokine effects on endothelial cells

HCAECs were maintained at the near confluent density 24 h before the exposure experiments and subsequently replaced with fresh media and incubated for another 24 h. At the fully confluent density, HCAECs were exposed to the following cytokines and subsequently incubated for another 24 h: IL-1β (R&D Systems, MN, USA) (1.1 nM), TNF-α (R&D Systems) (0.3 nM or 10 nM), IFN-β (PeproTech Inc., NJ, USA) (0.03 nM or 1.3 nM), IFN-γ (R&D Systems) (0.04 nM or 0.5 nM), OSM (R&D Systems) (1.1 nM or 2.7 nM). The cells were treated at two different concentrations; one was slightly over the median effective dose (ED_{50}) specified by the manufacturers and other was excessively over the ED_{50}. As negative controls for exposure (mock samples), fresh medium containing no cytokines were added to the culture.

2.3. Poly A(+) RNA preparation

The cells were harvested 24 h after exposure to cytokines with TRIzol Reagent (Invitrogen Corp., CA, USA). The cell lysates obtained were subjected to total RNA extraction according to the manufacturer's instructions. Subsequently, total RNA was subjected to polyA(+) RNA isolation with a MicroPoly(A) Purist Kit (Ambion Inc., TX, USA) in accordance with the manufacturer's instructions. Eventually, polyA(+) RNA was divided into aliquots of 2 μg, precipitated with ethanol, and stored at −20 °C.

2.4. Preparation of DNA microarrays and acquisition of gene expression profiles

A set of synthetic polynucleotides (80-mers) representing 22512 species of human transcript sequences that are mostly originated from the Reference Sequence (RefSeq) project clones deposited in the National Center for Biotechnology Information (NCBI) database was purchased (MicroDiagnostic, Tokyo, Japan) and printed on a glass slide (coated glass slide for the microarray, type I; Matsunami Glass Ind., Ltd., Kishiwada, Japan) with a custom-made arrayer (designated as the 22K array) [11,12]. Two micrograms of poly(A)+ RNA were labeled with SuperScript II (Invitrogen Corp.) and cyanine 5-deoxyuridine triphosphate (dUTP) (Perkin–Elmer Inc., MA, USA) for each HCAECs sample or cyanine 3-dUTP (Perkin–Elmer Inc.) for a human common reference RNA. The human common reference RNA was prepared by mixing equal amounts of poly(A)+ RNA extracted from 22 cell lines (A431, A549, AKI, HBL-100, HeLa, HepG2, HL60, IMR-32, Jurkat, K562, KP4, MKN7, NK-92, Raji, RD, Saos-2, SK-N-MC, SW-13, T24, U251, U937, and Y79). Hybridization and subsequent washes of the arrays were performed with a Labeling and Hybridization Kit (MicroDiagnostic). Hybridization signals were measured with a GenePix 4000A scanner (Axon Instruments Inc., Union City, CA) and then processed into primary expression ratios (ratios of cyanine 5 intensity of each sample to cyanine 3 intensity of the human common reference RNA) by the GenePix Pro 3.0 software (Axon Instruments Inc.). Normalization was performed for each ratio by multiplying the normalization factors calculated by the GenePix Pro 3.0 software. The primary expression ratios were converted into $\log_2$ values (designated as log ratios). All the data in accordance with the MIAME guideline were deposited at DDBJ via CIBEX (http://cibex.nig.ac.jp/index.jsp) in Accession Numbers CBX14.

2.5. Data analysis

Data processing and subsequent hierarchical clustering analysis were conducted with an MDI gene expression analysis software package (MicroDiagnostic). To compare the cytokine-treated samples against the mock-treated samples (negative controls) for each cytokine, log ratios obtained for the mock-treated samples were subtracted from the log ratios obtained for each cytokine-treated sample (designated as relative log ratios) (Supplementary Table 1). Next, genes, the relative log ratios of which in the two independent samples for each cytokine were greater than 0.75 or smaller than −0.75, were extracted (Supplementary Table 2). In order to obtain data on these extracted genes, they were processed into a matrix (rows, genes; columns, samples) and subjected to two-dimensional hierarchical clustering analysis. Furthermore, for extracting genes that enabled us to distinguish between the two groups of samples in terms of the presence or absence of the expression alteration of interest (designated as the presence group and the absence group, respectively), we calculated a statistical value consisting of the absolute value of the difference between the mean average of relative ratios among the presence group and that among the absence group divided with the sum of the standard deviation of the relative log ratios among the presence group and that among the absence group (designated as signal:noise ratios); this was done for all two-group combinations among the five individual cytokines. Eventually, we extracted genes that revealed signal:noise ratios that were greater than two and absolute values of the relative log ratios for the presence groups that were greater than 0.75.

3. Results

3.1. Acquisition of gene expression profiles

To investigate the heterogeneity of the responses of endothelial cells, which line the blood vessels and are directly exposed to a variety of stimulatory factors in the blood, to cytokine stimulation in terms of alteration in gene expression, we obtained comprehensive gene expression profiles with DNA microarrays containing about 22000 species of probes for human transcripts from HCAECs that were treated for 24 h with the cytokines that are responsible to a variety of cellular functions. In this study, we chose the following five cytokines as models: IL-1β and TNF-α, both involved in inflammatory responses following infection and tissue damages [13]; IFN-β and IFN-γ principal mediators in immune responses such as antiviral activities and antitumorigenic activities [14,15]; and OSM, involved in accelerative and inhibitory functions in inflammation and in vascularization [16–19]. These cytokines were exposed to HCAECs at two different concentrations in two independent experiments to eliminate the genes in which the expression levels are robustly affected by the differences in the magnitude of stimulation.

3.2. Overview of alteration of gene expression in HCAECs after cytokine stimulation

First, we extracted genes in which both relative log ratios (see Section 2) in two different samples treated with identical

Table 1
The number of genes that exhibited the alteration of expression levels after individual cytokines

Treatment	Upregulated[a]	Downregulated[b]	Total
TNF-α	407	184	591
IL-1β	181	88	269
IFN-β	121	28	149
IFN-γ	129	28	157
OSM	46	33	79

[a]The number of increased genes that showed relative log ratio greater than 0.75 across two independent samples treated with individual cytokines.
[b]The number of decreased genes that showed relative log ratio smaller than −0.75 across two independent samples treated with individual cytokines.

cytokines were greater than 0.75 or smaller than −0.75; this was done to concentrate only on the genes that were robustly affected by the cytokine stimulation. The numbers of genes extracted under the abovementioned condition were: TNF-α, 591; IL-1β, 269; IFN-β, 149; IFN-γ, 157; OSM, 79 (Table 1). These results indicate that the most robust influence was induced by TNF-α and the faintest influence was produced by OSM at the gene expression level in HCAECs. Next, to compare the cellular responses in HCAECs at the gene expression level, we generated a data matrix with the genes described above and subjected it to a two-dimensional hierarchical clustering analysis (Fig. 1). The clustering analysis provided five different sample clusters and each cluster consisted of two samples treated with identical cytokines (Fig. 1). Moreover, the TNF-α and IL-1β clusters formed an identical larger cluster; the IFN-β and IFN-γ clusters generated an identical larger cluster. These two larger clusters produced one of the largest clusters in the dendrogram (Fig. 1). These results are consistent with the previously reported findings that a pair of TNF-α and IL-1β or that of IFN-β and IFN-γ share similar activities in terms of the alteration of gene expression and functional responses [20,21]. Moreover, we observed that the alteration of gene expression in OSM-treated HCAECs was less robust than those in the other four cytokine-treated cells and that the constituents of the genes in which expression altered after the OSM exposure were less overlapped compared to those found in the other four cytokine-treated samples. Taken together, these findings indicate that a parallel analysis of genes extracted from expression profiles clearly distinguishes the responses in the five cytokine-treated HCAECs in a cytokine-specific manner.

3.3. Identification of genes that shared similar response patterns among the different cytokine-treated samples

For the clustering analysis shown in Fig. 1 in the direction across genes, we paid attention to the presence of genes in which expression altered in multiple and single cytokine-specific manners. The reason is that the response patterns for the alteration of gene expression should reflect the extent of specificity and similarity in the responses of HCAECs against these cytokines. Therefore, we sought to disclose the presence of more minutely heterogeneous response patterns among the five cytokines compared to those brought forth by the hierarchical clustering analysis (as shown in Fig. 1) and to identify the genes included in the response patterns. For this advanced purpose, we extracted genes from the data matrix of relative

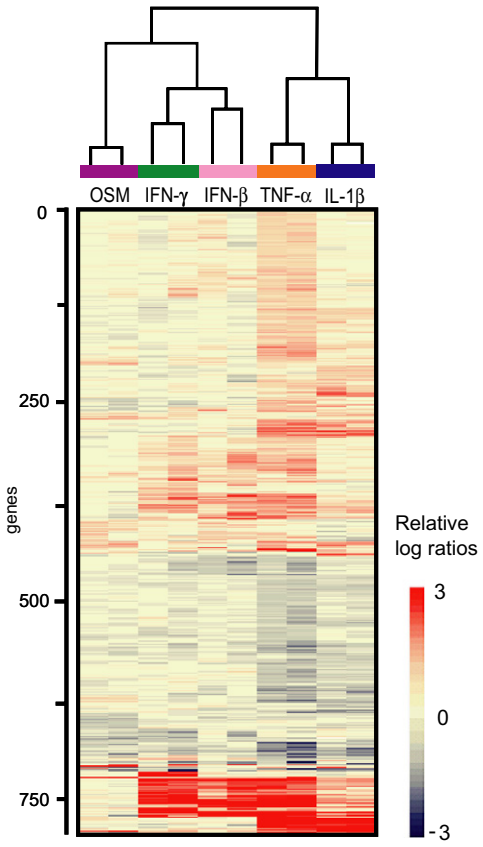

Fig. 1. Comparison of gene expression profiles obtained from HCAECs stimulated with cytokines. Robustly expressed genes after stimulation with IL-1β, TNF-α, IFN-β, IFN-γ, and OSM were extracted and subjected to two-dimensional hierarchical clustering analysis. Columns and rows indicate samples (10) and genes (789), respectively. Genes and samples are aligned in the order defined by the results of the clustering analysis. The dendrogram indicates the relationship among the samples based on dissimilarity coefficients calculated through the clustering analysis. The color bar at the bottom of the figure represents the grades of the relative expression levels: increase, red; decrease, blue. Each color box under the dendrogram at the top of the figure depicts an individual cytokine: IL-1β, blue; TNF-α, orange; IFN-β, pink; IFN-γ, green; OSM, violet. All the relative log ratios included in this figure are shown in Supplementary Table 2.

log ratios by another statistical procedure; these genes were included in all the two-group combinations of the five cytokines (see Section 2).

The statistical extraction provided 15 species, each with distinct patterns (designated as response patterns) (Table 2). Out of these 15 patterns, 10 response patterns were common to multiple cytokines (response patterns A, B, C, D, I, J, K, L, M, and N), whereas five response patterns were solely specific to a single cytokine for all the five cytokines tested (response patterns E, F, G, H, and O). Among the response patterns solely specific to individual cytokines, the most prominent response pattern was observed in a TNF-α-specific manner as response pattern E consisting of 95 genes. On the other hand, the most quiescent response pattern was seen in an IFN-β-specific manner as response pattern O consisting of one gene (Table 2).

The extracted 15 response patterns included identical genes in duplicate across two response patterns. Response patterns D and J included *PLSCR1* in which expression increased

Table 2
Response patterns and the number of genes included in the response patterns

Response pattern	TNF-α	IL-1β	IFN-β	IFN-γ	OSM	Genes[a]
A	+	+	+	+		39
B	+	+				55
C	+		+	+		9
D	+		+			7
E	+					95
F		+				28
G				+		19
H					+	10
I	+		+	+	+	1
J		+		+	+	1
K	+	+		+		1
L	+			+		1
M	+				+	1
N		+			+	1
O			+			1

Notes: +, the presence of alteration (a constituent of the presence group). *Blank*: the absence of alteration (a constituent of the absence group). The meaning of alteration is as follows: (i) in the presence group, the absolute value of the relative log ratio was greater than 0.75 for both of the two independent samples treated with individual cytokines; (ii) the absolute value of the difference in the mean average of the relative ratios divided by the sum of the standard deviation of the relative ratios among each group for individual cytokines was greater than 2.
[a]The number of genes constituting each response pattern.

after stimulation with all five cytokines. Response patterns B and E included five genes (*LTB*, *LAMC2*, *TNFAIP3*, *SDCCAG28*, and *D6S49E*) in common; response patterns B and F included four genes (*CLU*, *SCYB5*, *SART-2*, and *MYO1B*) in common (all the full names of the genes are shown here and below, while abbreviations are listed in Supplementary Table 2).

Moreover, seven response patterns contained a single gene as the constituent (response patterns I, J, K, L, M, N, and O). These response patterns involved *PLSCR1* in response pattern J, *GAGED2* in response pattern I, *TNFAIP2* in response pattern K, *AKR1C3* in response pattern L, *LOC58489* in response pattern M, *IER3* in response pattern N, and *COLF6967* in response pattern O (Supplementary Fig. 1). Since these patterns include a single gene only as each constituent, further accumulation of experimental data obtained with a variety of exogenous stimuli to multiple cell species and information on gene ontology should be required to speculate the relevance and biological significance of these patterns.

3.4. Genes in which expression altered similarly in a multiple cytokine-specific manner

To study the kind of cellular responses represented by the response patterns consisting of two and more genes (response patterns A, B, C, D, E, F, G, and H), we analyzed the association between the previously accumulated findings and the individual genes included in the response patterns. First, we confirmed that all the response patterns exhibited the two largest clusters that should clearly distinguish the two groups of interest among all the samples by hierarchical clustering analysis in the direction across the samples (Figs. 2 and 3). Next, for genes encoding for actual proteins among those consisting of these response patterns, we scrutinized the definition of biological process in Gene Ontology Consortium (GO) (http://www.geneontology.org/) edited by Human Protein Reference Database (http://www.hprd.org/) [22] and reference information.

These results indicate that the cellular responses of HCAECs include at least four different response patterns defined by the alteration of the expression levels of genes, as shown in Fig. 2, and that each response pattern contains genes that are affected differently by two groups of cytokine, i.e., the group for the presence of influence (the robustly influential group) and the one for the absence of influence (the weakly influential group), which comprises at least two cytokines.

3.5. Genes in which expression levels solely altered in a single cytokine-specific manner

We continued an identical approach to characterize the response patterns comprising genes in which expression levels solely altered in a single cytokine-specific manner.

Fig. 3A shows the response pattern E consisting of genes that exhibited robust alteration only after TNF-α stimulation. Fig. 3B represents the response pattern F consisting of genes that exhibited robust alteration only after IL-1β stimulation. Fig. 3C displays the response pattern G consisting of genes that exhibited robust alteration only after IFN-γ stimulation. Fig. 3D demonstrates the response pattern H consisting of genes that exhibited strong alteration only after OSM stimulation, including those that showed different expression changes amongst each other in case of both OSM stimulation and IL-1β and TNF-α stimulation and those that showed alteration only after OSM stimulation.

These results indicate that cellular responses of HCAECs include at least four different response patterns defined by the alteration of expression levels of genes shown in Fig. 3 and that each response pattern contains genes solely affected by a single cytokine. Furthermore, these results suggest the presence of greatly different modulation mechanisms in HCAECs for the OSM stimulation and the IL-1β and TNF-α stimulation pathways.

3.6. Comparison of 15 response patterns in parallel

Eventually, we sought to characterize all 15 response patterns and compare them to one another by connecting the number of genes belonging to each criterion in the biological process to each response pattern (Table 3). Consequently, we obtained the following findings. Genes that are well characterized in function and involved in immune response following viral and bacterial infections were included abundantly in the response patterns A (12 out of 39; 31%) and G (7 out of 19; 37%) but scarcely in the response patterns E. Genes associated with apoptosis were included in the response patterns A, B, and E. Genes involved in cell growth/maintenance were present in the response patterns B, E, F, and H but absent in the other response patterns. Genes related to protein metabolism were present in the response patterns A, B, C, D, E, and G but absent in the other response patterns. Between the response patterns A and D and the response pattern B, we observed contrastive characteristics with respect to protein metabolism; the response patterns A exhibited an increased expression of constituents belonging to the ubiquitin–proteasome system, including

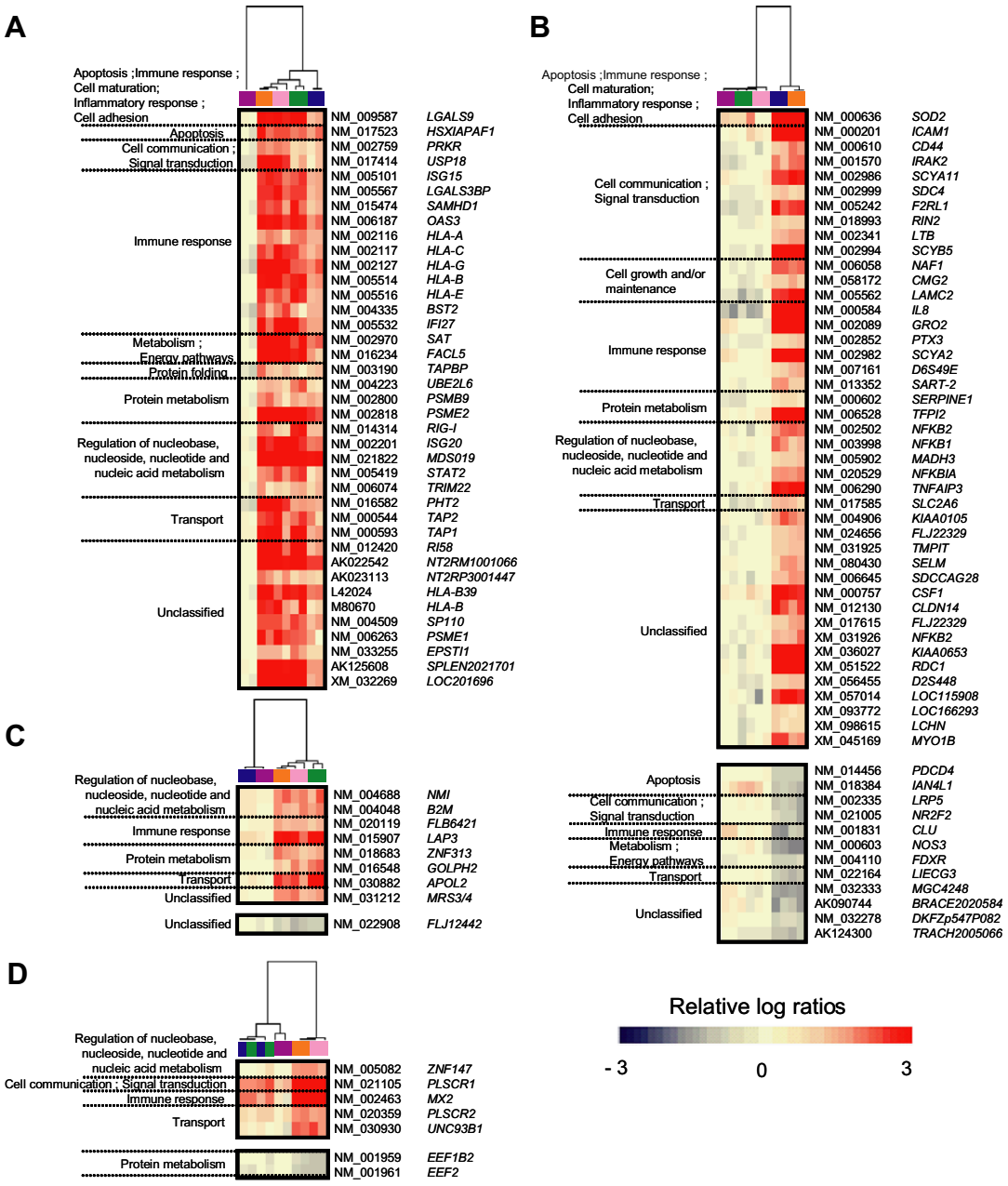

Fig. 2. Genes in which expression levels altered similarly in a multiple cytokine-specific manner. (A) Response pattern A consisting of 39 genes in which expression levels altered similarly after stimulation with TNF-α, IL-1β, IFN-β, and IFN-γ. (B) Response pattern B composed of 55 genes that exhibited similar alteration of expression levels after stimulation with TNF-α and IL-1β. (C) Response pattern C comprising nine genes that exhibited similar alteration of expression levels after stimulation with TNF-α, IFN-β, and IFN-γ. (D) Response pattern D containing seven genes in which the expression levels altered similarly after TNF-α and IFN-β stimulation. The color bars at the bottom right of the figure represents the grades of the relative expression levels: increase, red; decrease, blue. Each color box at the top of the figure depicts an individual cytokine: IL-1β, blue; TNF-α, orange; IFN-β, pink; IFN-γ, green; OSM, violet. At the left side of each panel, the biological process for each gene contained is indicated, which is defined in the Gene Ontology Consortium (http://www.geneontology.org/). At the right side of each panel, the accession number and gene ID for each gene contained is demonstrated, which is derived from the National Center for Biotechnology Information (NCBI) Reference Sequences (http://www.ncbi.nlm.nih.gov/RefSeq/). The full name of each gene is shown in Supplementary Table 2, linked to each Gene ID. All the relative log ratios included in this figure are shown in Supplementary Table 3.

PSMB9 and *PSME2*, and the response pattern D showed the decreased expression of elongation factors, namely, *EEF2* and *EEF1B2*, whereas the response pattern B exhibited an increased expression of an inhibitory factor for proteolysis, *SERPINE1*.

Overall, these results indicate that out of the 15 response patterns in which at least one gene was successfully extracted through the statistical approach, 11 response patterns were differentially characterized by representing the number of genes contained in individual criteria of the biological process in the GO only. These suggest that the approach to link the genes constituting the response patterns with the biological process defined by the GO may endorse the classification with the response patterns as being biologically meaningful.

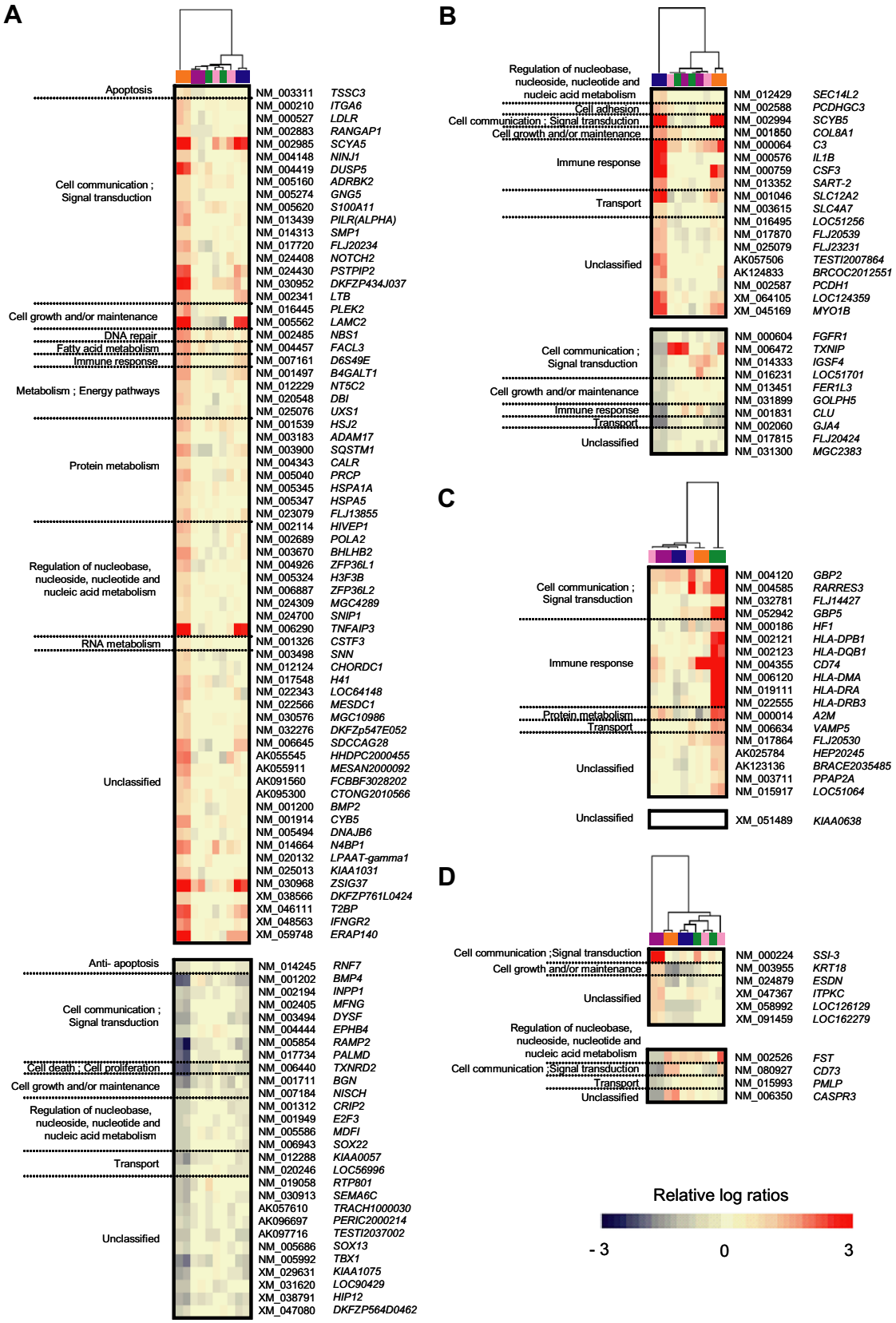

Fig. 3. Genes in which expression levels solely altered in an individual cytokine-specific manner. (A) Response pattern E consisting of 95 genes that exhibited expression alteration only after TNF-α stimulation. (B) Response pattern F comprising 28 genes that demonstrated expression alteration only after IL-1β stimulation. (C) Response pattern G composed of 19 genes that exhibited expression alteration only after IFN-γ stimulation. (D) Response pattern H consisting of 10 genes that showed expression alteration only after OSM stimulation. All the relative log ratios included in this figure are shown in Supplementary Table 4.

Table 3
Distribution of the number of genes assigned to the major criteria of the biological process defined by the Gene Ontology Consortium (GO)

Biological process[a]	Response pattern														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Anti-apoptosis					1										
Apoptosis	2	3			1										
Cell adhesion	1	1				1									
Cell communication; signal transduction	2	11		1	23	5	4	2		1					
Cell death					1										
Cell growth and/or maintenance		3			4	3		1							
Cell maturation	1	1													
Cell proliferation					1										
DNA repair					1										
Fatty acid metabolism					1										
Immune response	12	8	2	1	1	5	7								
Inflammatory response	1	1													
Metabolism; energy pathways	2	2			4							1			
Protein folding	1														
Protein metabolism	3	2	2	2	8		1								
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	5	5	2	1	13	1		1							
RNA metabolism					1										
Transport	3	2	1	2	2	3	1	1							
Unclassified/biological_process unknown	1	6	1		10	5	5				1				
Unclassified/unannotated	9	14	1		24	5	1	5	1				1	1	1

[a]Each gene is assigned to all the corresponding criteria of the biological process defined by GO (http://www.geneontology.org/) in a repetitive manner.

4. Discussion

In this study, we compared in parallel the cellular response of HCAECs to the cytokines, such as IL-1β, TNF-α, IFN-β, IFN-γ, and OSM, which are profoundly associated with the immune and inflammatory reactions in endothelial cells from the standpoint of the comprehensive alteration in gene expression. We conducted two independent approaches to analyze expression profiles obtained from HCAECs stimulated with these cytokines. One was hierarchical clustering analysis done only with genes in which expression levels altered robustly after the stimulation; the other was statistical extraction of the genes of interest from all the profiles divided into all combinations of two groups among five cytokines. Based on the results of the clustering analysis in the direction across samples, we concluded that these genes are responsible for distinguishably classifying these five cytokines at the gene expression level. Moreover, based on the results of the statistical extraction of the genes, we concluded that cellular responses after stimulation with these five cytokines solely described by gene expression ratios were composed of at least 15 patterns (designated as response patterns) (Table 2), identifying genes that constitute the individual response patterns. Eventually, we linked these genes constituting the response patterns with the biological process defined by the GO and endorsed the classification with the response patterns as being biologically meaningful (Table 3).

As an experimental model system to investigate the responses of endothelial cells to a variety of exogenous stimuli, we chose five cytokines associated with inflammation and HCAECs under a flat culture condition. Since HCAECs are reported to exhibit different responses to inflammation cytokines when compared to human umbilical vein endothelial cells and human pulmonary artery endothelial cells in terms of gene expression and protein secretion [23,24], it may be appropriate that the implication of our findings presented here should be restricted to HCAECs as a model. Further experiments should be required with other subtypes of endothelial cells in addition to different lots of HCAECs under distinct culture conditions such as those that allow endothelial cells to form tubular structures to establish more general implication of endothelial cell responses to the cytokines used in this study. Moreover, although we chose a single time point of 24 h after cytokine exposure since we failed to obtain robust alteration at the gene expression level in preliminary experiments with smaller microarrays at the time point of 4 h and 12 h after treatment (data not shown) and we could not observe obvious differences between expression profiles obtained from two independent samples treated with an identical cytokine (data not shown), data extensively obtained at earlier and later time points at multiple concentrations of cytokines may provide findings to reveal the kinetics of modulation in gene expression after the cytokine stimulation.

The results shown here indicate that our approach of analyzing the cellular responses of HCAECs to five individual cytokines from the novel standpoint of similarity and diversity in the alteration of gene expression levels is capable of identifying new description and classification of the constituents of the cellular response. Previously, several studies were conducted with DNA microarrays for analyzing the alteration of gene expression in endothelial cells after stimulation with cytokines [5–7]. Among them, the most minutely conducted study reported on the identification of the expression levels that altered in a TNF-α and IFN-γ-specific manner from the expression profiles obtained with five species of endothelial cells and three individual inflammatory cytokines (TNF-α, IFN-γ, and IL-4), a total of 15 individual samples analyzed in parallel [6]. In addition, the authors paid attention to the tissue specificity of endothelial cells and the functional specificity among three individual cytokines and isolated marker genes for individual cytokine function in respective species of endothelial cells. Similar to this report, most of

the previous studies focused on identifying markers specific to stimulation or to tissues and cells. In contrast, we at first focused on analyzing minutely the cellular responses solely described with gene expression ratios prior to identifying the specific marker genes. The 15 response patterns found in this study (response patterns A∼O) consist of genes belonging to various categories in the biological process outlined by GO in a response pattern-specific manner (Table 3). Thus, it is possible to remark that these response patterns are characterized by itself with a constitution of the numbers and species of genes included.

In this study, we chose cytokines as representative factors to stimulate endothelial cells. Endothelial cells respond to cytokines present in local microenvironments through altering gene expression. The cellular response induces a variety of alterations such as the promotion of the recruiting of immune cells via surface molecules expressed on the endothelial cells, increase in vascular permeability, and proliferation or apoptosis of the endothelial cells themselves [25–28]. However, endothelial cells should respond not only to secretory factors like cytokines but to humoral factors that derived exogenously such as endotoxins [29] and physical factors such as shear stress [30]. Therefore, in order to understand comprehensively the diversity of the response of endothelial cells, it is essential to systematically study the responses induced by diverse stimulations including physical, chemical, and biological factors. To achieve these systematic collections of cellular samples after various stimulations, it should be inevitable to comparatively analyze in parallel a great number of expression profiles on an identical platform. Our microarray system used in this study may contribute to establishing such a systematic analysis for the response of endothelial cells to the diverse stimulation since our system successfully analyzed 130 expression profiles in parallel [12].

Dysfunctions of endothelial cells are profoundly associated with a variety of pathological processes such as disseminated intravascular coagulation [31] and tumor progression [32]. Although focused studies have been conducted on these diseases recently, the pathological mechanism of these diseases and the response of endothelial cells involved in the mechanism are not fully understood. Comparative analysis in parallel for comprehensive gene expression profiles obtained with DNA microarrays from endothelial cells enables us to investigate the response of endothelial cells to a variety of exogenous stimulations from an extensive and minute standpoint. We expect that it may be contributable to the analysis of genes in such a way that it facilitates the understanding of the response of endothelial cells that are peculiar to vascular diseases and help in the invention of novel therapeutic targets for these diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.041.

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