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Inflammation Research

Gene expression profiling of Ca^{2+}-ATPase inhibitor DTBHQ and antigen-stimulated RBL-2H3 mast cells

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Abstract. *Objective and Design:* Ca^{2+} signaling is critical for mast cell activation by antigen stimulation, and we previously described that the signaling can be mimicked by Ca^{2+}-ATPase inhibitors. We therefore investigated the effect of the Ca^{2+}-ATPase inhibitor and antigen stimulation on the gene expression profiles of RBL-2H3 mast cells.
Material: A Ca^{2+}-ATPase inhibitor, 2,5-di(*tert*-butyl)-1,4-hydroquinone (DTBHQ), an antigen (dinitrophenylated BSA), a high-density oligonucleotide microarray (Affymetrix GeneChip) technique, and a well-characterized rat mast cell line RBL-2H3 were used.
Treatment: RBL-2H3 cells were activated for 3 h with 10 μM DTBHQ, which increases cytosolic Ca^{2+} concentration, or 10 μg/ml antigen, which cross-links IgE receptors, and the mRNA expression profiles (8,799 genes) were analyzed with GeneChip arrays (n = 3).
Methods: Expression levels were measured by GeneChip, and the differences were tested by Welch's t-test and P-values less than 0.05 were considered statistically significant. Values are expressed as means ± SEM.
Results: The genes, including MCP-1, GADD45, Relaxin H1, CSF-1, c-jun-oncogene, Pyk-2, NKR-P2 and CREM, were significantly up-regulated by both DTBHQ and antigen stimuli, whereas the genes including interleukin (IL)-3, IL-4, IL-9, IL-13, GADD153, butyrate response factor, and Fas ligand, were up-regulated by DTBHQ alone. On the other hand, the expression of several genes, including GATA-1, were down-regulated by DTBHQ stimulation.
Conclusions: These results suggest 1) that DTBHQ seems to induce proinflammatory responses by stimulating the production of several cytokines through the expression of several transcription factors, 2) that the changes in gene expression profile induced by DTBHQ and by IgE receptor cross-linking in mast cells were almost the same, but many more stress-inducible genes like GADD153 were up-regulated by the former.

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Key words: DNA microarray – Mast cell – Gene expression – Antioxidant – High-affinity IgE receptor

Introduction

When mast cells are activated by IgE and antigen, they not only rapidly secrete inflammatory mediators, such as histamine, but also produce a variety of cytokines or other biologically active molecules [1]. Thus, it is important to know the gene expression profile of mast cells to search for novel responsive genes in allergic responses.

The RBL-2H3 cell line, which has been established from rat basophilic leukemia cells [2, 3], and has many of the characteristics of mucosal mast cells, has been used as a model system for IgE-dependent and -independent mast cell activation [4–6]. An earlier work by Chen et al. using the SAGE method investigated up- and down-regulated gene tags in RBL-2H3 cells following antigen stimulation [7].

In this study, we analyzed the gene expression profile of RBL-2H3 mast cells by high-density oligonucleotide microarray technology, in the form of the GeneChip®. The key principle of this technology is hybridization of complementary RNA (cRNA) to high-density oligonucleotide probes synthesized by photolithography [8].

The GeneChip technology has recently generated a great deal of information about gene expression profiles in many organs for many purposes, for examples, cell cycle analysis [9, 10], identifying B-cell lymphoma [11], and many cancer researches [12, 13]. Although a few studies of human mast cells have been conducted of the GeneChip [14, 15], there have been no reports of use of this technology in studies on RBL-2H3 mast cells.

Cross-linking of high-affinity IgE receptors (FcεRI) on a mast cell by IgE and antigen leads to protein tyrosine phosphorylation, Ca^{2+} signaling, PI turnover, degranulation of histamine, and production of several cytokines or chemokines [16–18]. On the other hand, we have recently investigated the effect of several inhibitors of endoplasmic reticulum Ca^{2+}-ATPase (Ca^{2+} pumps) on mast cell activation

[19], they cause Ca^{2+} leakage into the cytoplasm, influx of Ca^{2+} from the extracellular medium via activation of Ca^{2+}-release activated channels, and production of several cytokines [19]. The results of using these drugs have suggested that two types of mediator-releasing processes in RBL-2H3 mast cells: (1) a process in which an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) is sufficient to release mediators (e.g., leukotriene C4, interleukin-4, and MCP-1 production), and (2) a process in which both an increase in $[Ca^{2+}]i$ and activation of protein kinase C are needed to release mediators (e.g., TNF-α production and degranulation) [19]. Ca^{2+}-ATPase inhibitors seem to be good probes to use to study the mechanism of Ca^{2+}-related signal transduction and proinflammatory responses in mast cells. We chose one typical Ca^{2+}-ATPase inhibitor, the antioxidant 2,5-di(*tert*-butyl)-1,4-hydroquinone (DTBHQ) [20] to use in this study. To better understand the mechanisms of the proinflammatory responses of RBL-2H3 cells to DTBHQ, we used GeneChip technology to investigate the changes in gene expression induced by exposure to this antioxidant. We also analyzed changes in the gene expression of RBL-2H3 cells stimulated with antigen (IgE-receptor cross-linking) and compared them with the changes induced by DTBHQ.

Materials and methods

Reagents, buffer and cells

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Co. (Tokyo, Japan), and fetal bovine serum was from Sigma (St. Louis, MO). Murine anti-dinitrophenyl (DNP) monoclonal IgE antibody (IgE-53-569) and dinitrophenylated BSA (DNP_7-BSA) were prepared as described previously [21]. TRIzol reagent and SuperScript Choice system were purchased from Invitrogen Corp (Carlsbad, CA). TaqMan Gold RT-PCR kit and TaqMan Universal PCR Master Mix were purchased from Applied BioSystems (Foster City, CA). RBL-2H3 cells were a kind gift of Dr. R. P. Siraganian (National Institute of Dental Research, National Institutes of Health, Bethesda, MD), and the cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum at 37°C under 5% CO_2 in a humidified atmosphere, as described previously [21].

For stimulation, RBL-2H3 cells (1×10^7) were sensitized with 1:800 diluted anti-DNP IgE overnight, and after washing twice with DMEM, they were stimulated with 10 µg/ml DNP_7-BSA or 10 mM DTBHQ at 37°C for 3 h or the period indicated [21]. To compare the expression levels in resting and activated RBL cells we chose the stimulation interval 3 h, because the expression of MCP-1 reached maximum at 3 h after antigen- or DTBHQ-stimulation [22].

GeneChip analysis

Total RNA from resting or activated RBL-2H3 cells (1×10^7) was prepared with a TRIzol reagent according to the manufacturer's instructions. A 20 µg of total RNA was used for each experiment, and the target cRNA for Affymetrix GeneChip analysis was prepared according to the manufacturer's instructions. Affymetrix GeneChip Rat Genome U34A arrays consisting of 8,799 high-density oligonucleotide probe sets were hybridized with the targets at 45°C for 16 h, and then washed and stained by using the GeneChip Fluidics Station. The arrays were scanned with the GeneArray scanner (Affymetrix), and the fluorescence images obtained were processed by the Expression Analysis algorithm in Affymetrix Microarray Suite (ver. 4.0) and Microsoft Excel. The Average Difference (Avg Diff) was obtained and the average of the Avg Diff was scale-adjusted to 2,500 by using the Microarray Suite algorithm. If the Avg Diff value was below the raw Q value, it was replaced with the raw Q value by Microsoft Excel [10].

The data were imported into GeneSpring® analysis software (ver. 4.1.3, Silicon Genetics, Redwood City, CA) for further analysis. All expression signals (Avg Diffs) were normalized across the all genes and all arrays by the GeneSpring standard normalization algorithm. Genes that showed substantial up- or down-regulation after stimulation were then selected from three independent experiments based on Welch's t-test, and the genes whose maximum value was less than 1,000 (one-third of Avg Diff of an external control BioB), the genes whose fold change was less than 3, and the gene tags not fully identified (Expressed Sequence Tags; ESTs) were excluded from the gene list. If someone needs the entire gene list, please e-mail to the corresponding author.

Real-time PCR analysis

RBL-2H3 (1×10^7) cells were at 37°C stimulated with antigen (10 µg/ml) for 0, 1, 3, 6, 12 h or with DTBHQ (10 µM) for 3 h, and then harvested to prepare total RNA with TRIzol reagent. The amounts of MCP-1, GADD45, and GAPDH mRNA were measured by quantitative PCR using an ABI PRISM™ 7700 sequence detector (PE Applied BioSystems, Foster City, CA) according to the manufacturer's instructions. In each experiment, 100 ng total RNA, 0.3 µM forward primer, 0.3 µM reverse primer, and 0.3 µM fluorescent probe were used. The PCR conditions were: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min.

The primers and probes used for TaqMan analysis were: MCP-1 forward primer, 5′-ACT CAC CTG CTG CTA CTC ATT CAC T-3′; MCP-1 reverse primer, 5′-TGC TGC TGG TGA TTC TCT TGT AGT-3′; MCP-1 TaqMan probe, 5′-VIC-TCC AGC CGA CTC ATT GGG ATC ATC TT-Tamra-3′; GADD45 forward primer, 5′-TGG CTG CGG ATG AAG ATG A-3′, GADD45 reverse primer: 5′-TCG CAA CAG AAA GCA CGA AT-3′; GADD45 TaqMan probe, 5′-VIC-TCT GCA ACC ACT CCC TC-Tamra-3′. For GAPDH and IL-3, we used TaqMan Rodent GAPDH Control Reagents VIC™ Probe #4308313 and Pre-Developed TaqMan Assay Reagents Rat IL-3 MGB #4319384F, respectively.

Statistical analysis

Differences were tested by Welch's t-test based on three independent experiments, and P-values less than 0.05 were considered statistically significant. Values are expressed as means ± SEM.

Results

Global analysis of gene expression profiles in RBL-2H3 cells

As described previously [20, 22], when the RBL-2H3 cells were stimulated with 10 µg/ml antigen (DNP_7-BSA) and 10 µM DTBHQ, the intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) of the cells rapidly increased from 62 nM to 160 nM and 220 nM, respectively, and the increase in $[Ca^{2+}]i$ induced by DTBHQ was sustained longer than the increase in response to the antigen. Affymetrix GeneChip U34A arrays were then used to analyze global transcripts in RBL-2H3 cells under the same conditions, and we had previously determined that the conditions were not toxic for the cells, as determined by LDH-cytotoxicity assay [23]. The U34A array contains 8,799 probes derived from all full-length or anno-

tated rat genes as well as thousands of rat EST clusters, including some redundant probes specific to an other part of the identical transcript. This allows aproximately 5,000 individual transcripts to be analyzed simultaneously with this array.

The results showed that 1.8% (162/8,799) of the genes and ESTs included in a U34A array were up-regulated after antigen stimulation and 1.5% (130/8,799) were down-regulated (Fig. 1). Similarly, 2.8% (248/8,799) were up-regulated after DTBHQ stimulation, and 4.0% (355/8,799) were down-regulated. Both stimuli induced up-regulation of 67 genes in common and down-regulation of 60 genes in common.

In Fig. 1, the transcripts significantly up- or down-regulated by antigen stimulation are shown in green, those up- or down-regulated by DTBHQ stimulation in red, and those up- or down-regulated by both, in yellow. The vertical axis represents the expression level normalized to the median value across all genes and all arrays using GeneSpring software. In this figure, the gene expression detected by redundant probes that are specific to the other part of an identical transcript are also included.

Up-regulated genes in RBL-2H3 cells

Chen et al. used the SAGE method and found that 34 genes were up-regulated 3.5 h after antigen stimulation while 9 were down-regulated in RBL-2H3 cells [7]. They listed 11 known genes and 23 gene tags not identified at that time. In the present study, we identified 162 and 248 transcripts up-regulated by antigen and DTBHQ stimulation, respectively (Fig. 1). As shown in Table 1, we selected 31 genes from the up-regulated genes by excluding some transcripts as described in Materials and methods.

As previously reported by Chen et al., MCP-1, relaxin H1, and CSF-1 were significantly up-regulated when RBL-2H3 cells by antigen stimulation for 3 h. The transcript that exhibited the greatest increase (as measured by Avg Diff change) was monocyte chemoattractant protein MCP-1 [24]. We also identified several up-regulated genes that have not been previously reported in mast cells, including GADD45, oxidized LDL receptor, NKR-P2 and PYK2. The growth arrest and DNA damage-inducible gene GADD45 [25, 26] was the second most increased transcript in our array experiments. All of the transcripts above were also significantly up-regulated by DTBHQ stimulation, but a few, including cytokine-inducible SH2-containing protein (Cish) and calcium-independent α-latrotoxin receptor homolog 3 (CIRL-3), were up-regulated by antigen stimulation alone.

DTBHQ stimulation, on the other hand, affected the expression of a wide range of genes (Fig. 1b). Like by antigen stimulation, the transcript most increased by DTBHQ stimulation was MCP-1, and the second was GADD45. Several transcripts, such as interleukin (IL)-3, IL-4, IL-9, IL-13, butyrate response factor 1, aryl hydrocarbon receptor (AhR), GADD153, Fas antigen ligand, and hexokinase II, were up-regulated only by DTBHQ stimulation.

Down-regulated genes in RBL-2H3 mast cells

In the previous study by Chen et al., 9 transcripts, including rat mast cell protease (RMCP)-5, were significantly decreased in RBL-2H3 cells after antigen stimulation [7]. In

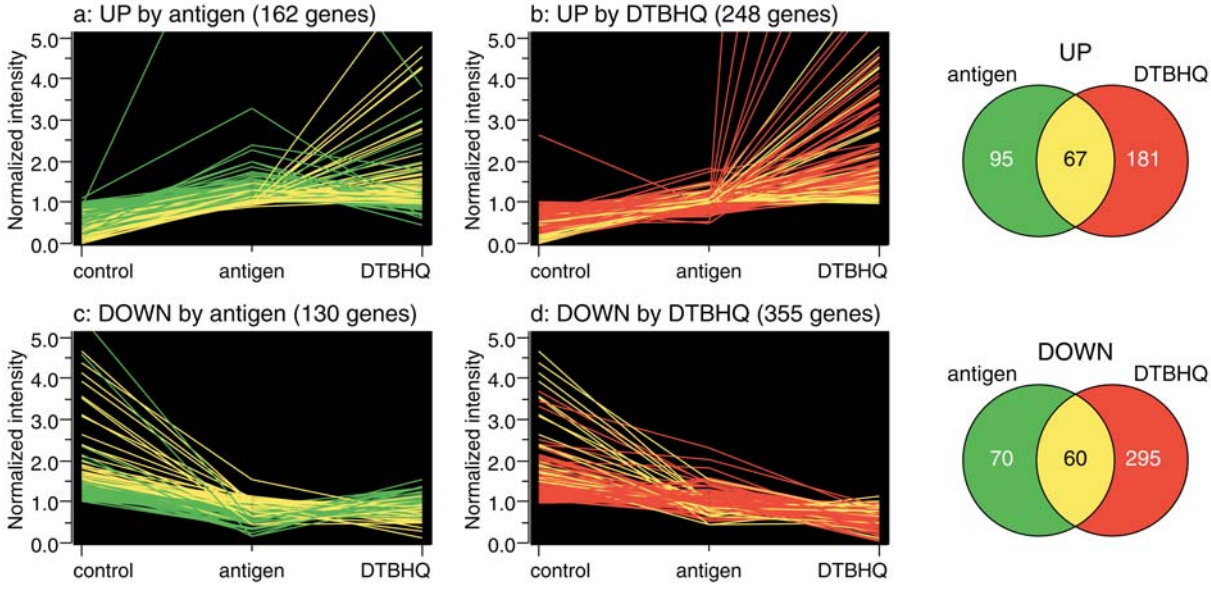

Fig. 1. Gene expression changes in RBL-2H3 mast cells. Global gene expression in resting cells and cells activated by 10 µg/ml antigen or 10 µM DTBHQ for 3 h was measured by using the GeneChip. The expression signals measured (Avg Diff) were normalized to the median value across all genes (n = 8,799) and all arrays (n = 9) by GeneSpring software. Genes whose expression was significantly different (* $p < 0.05$ by Welch's t-test) were selected and classified into 4 categories: **a**, up-regulated (UP) by antigen stimulation; **b**, up-regulated by DTBHQ stimulation; **c**, down-regulated (DOWN) by antigen stimulation; **d**, down-regulated by DTBHQ stimulation. The green, red, and yellow lines represent the genes whose expression levels was significantly changed after stimulation by antigen, DTBHQ, and both, respectively. The Venn diagrams represent the numbers of genes in the above categories. Three independent experiments were performed for each condition, but results are presented as mean without SD.

Table 1. Up-regulated genes in RBL-2H3 cells. The level of gene expression shown is the average difference (Avg Diff) obtained using GeneChip Microarray Suite software. The Avg Diff is an indication of hybridization to perfectly matched oligonucleotide probe sets versus hybridization to mismatched oligonucleotide probes. The statistical significance of differnces in gene expression levels between stimulated and control RBL-2H3 mast cells was assesed by Welch's t-test (*$p < 0.05$) based on data from three independent experiments. The genes whose maximum value was less than 1,000 and the genes whose fold change was less than 3 were excluded from the list. Values are mean ± SD.

Gene description	GenBank Acc No.	Control (Avg Diff ± SD)	Antigen (Avg Diff ± SD)	DTBHQ (Avg Diff ± SD)
Cytokine or hormone				
MCP-1	X17053	1366 ± 224	36426 ± 5714*	46893 ± 8033*
Relaxin 1 H1	J00780	7233 ± 634	24695 ± 3395*	26724 ± 3307*
IL-3	X03914	37 ± 31	660 ± 299	19436 ± 1596*
IL-13	L26913	1034 ± 250	1396 ± 395	5669 ± 2737*
CSF-1	M84361	579 ± 395	2884 ± 318*	3502 ± 1579*
IL-4	X16058	19 ± 1	229 ± 186	5267 ± 385*
IL-9	L36460	291 ± 111	464 ± 329	3353 ± 379*
Growth arrest, cell death				
GADD45	L32591	13705 ± 1627	28102 ± 2957*	45346 ± 8101*
GADD153	U30186	1116 ± 300	1261 ± 247	7766 ± 1612*
Epithelial membrane protein-1	Z54212	19 ± 1	1380 ± 875	1304 ± 480*
Ink4 (CDK inhibitor 2b)	S79760	303 ± 209	621 ± 298	1141 ± 247*
Fas antigen ligand	U03470	48 ± 48	52 ± 48	1279 ± 51*
Transcription				
c-jun oncogene	X17163	1583 ± 729	3554 ± 1250*	6670 ± 2091*
Leucine zipper protein	M63282	1191 ± 305	1240 ± 200	5186 ± 554*
CREM	S66024	108 ± 112	791 ± 186*	3819 ± 204*
Aryl hydrocarbon receptor (AHR)	AF082125	62 ± 72	20 ± 1	1344 ± 355*
Signal transduction				
NR43 nuclear hormone receptor (NOR-1)	AI176710	22 ± 6	1292 ± 119*	7545 ± 1612*
Focal adhesion kinase-related protein (PYK2)	AF063890	823 ± 145	4037 ± 246*	4338 ± 141*
Cytokine-inducible SH2-containing protein (Cish)	AF065161	162 ± 204	2286 ± 648*	1761 ± 1632
Calcium-dependent tyrosine kinase	U69109	556 ± 285	1957 ± 902	1983 ± 755*
RDC-1 (orphan chemokine receptor)	AJ010828	577 ± 466	818 ± 128	2861 ± 1203*
Calcium-independent alpha-latrotoxin recepor homolog 3 (CIRL-3)	AF063103	329 ± 100	1332 ± 118*	1275 ± 381
Neuron-derived orphan receptor-2 (NOR-2)	X86003	388 ± 239	386 ± 57	1611 ± 49*
Cell adhesion regulator (CAR1)	U76714	231 ± 80	255 ± 145	1083 ± 126*
Others				
butyrate response factor 1	AI136891	3187 ± 1275	5347 ± 668	22753 ± 188*
tryptophan hydroxylase	X53501	800 ± 828	3286 ± 1122*	8108 ± 3405
NKR-P2	AF009511	511 ± 253	4223 ± 487*	3946 ± 1119*
progression elevated gene 3 protein (PEG-3)	AF020618	1187 ± 142	1670 ± 165*	4759 ± 442*
endothelial receptor for oxidized low-density lipoprotein	AI071531	225 ± 24	1174 ± 133*	3065 ± 1418*
hexokinase II	S56464	1000 ± 778	1211 ± 958	3351 ± 1104*
urokinase-type plasminogen activator	X63434	178 ± 163	1014 ± 204*	1726 ± 618

our present study, we identified 130 and 355 transcripts down-regulated by antigen and DTBHQ stimulation, respectively. As shown in Table 2, we selected 11 genes from the down-regulated genes by excluding some transcripts as described in Materials and methods.

Fos-related antigen, retinal protein RRG4, and A2b adenosine receptor were significantly reduced by both stimuli. After DTBHQ stimulation, many genes, including transcription factor GATA-1, TGFβ-inducible early growth response (Tieg), and hepatic squalene synthetase, were markedly down-regulated.

Unlike the results by Chen et al., RMCP-5 was not so significantly down-regulated, while RMCP-10 was reduced from 6839 ± 681 to 2512 ± 982, by antigen stimulation (data not shown).

Real-time PCR analysis for the up-regulated transcripts

To confirm our results obtained by microarray analysis, we performed a real-time PCR analysis (TaqMan PCR) to quantify the transcripts in RBL-2H3 cells. Fig. 2 shows the ratios of expression levels of MCP-1 and GADD45 mRNA to the house-keeping gene GAPDH, as a control. The levels of expression (ratios) of MCP-1 and GADD45 were 0.031 ± 0.003 and 0.169 ± 0.005, respectively, in resting cells (Fig.

Table 2. Down-regulated genes in RBL-2H3 cells. Statistically different genes (* $p < 0.05$, Welch's t-test) are listed as described in Table 1.

Gene description	GenBank Acc No.	Control (Avg Diff ± SD)	Antigen (Avg Diff ± SD)	DTBHQ (Avg Diff ± SD)
Growth arrest, cell death				
TGFB inducible early growth response (Tieg)	AI172476	1459 ± 324	1030 ± 479	225 ± 171*
Transcription				
transcription factor GATA-1	D13518	4769 ± 1319	1835 ± 826	897 ± 412*
Fos-related antigen	U34932	1401 ± 419	566 ± 311*	284 ± 123*
Signal transduction				
retinal protein (RRG4)	U40999	5764 ± 1055	2551 ± 420*	1849 ± 391*
interferon regulatory factor 1 (IRF-1)	M34253	2177 ± 660	1033 ± 99	378 ± 179*
A2b-adenosine receptor	M91466	1627 ± 317	778 ± 54*	348 ± 70*
LIMK-1	D31873	1857 ± 218	997 ± 75	685 ± 195*
regulator of G-protein signalling 12 PDZ-less variant (RGS 12)	AF035151	1045 ± 272	980 ± 845	164 ± 201*
Others				
UDP-Gal: glucosylceramide beta-1,4-glactosyltransferase	AF048687	1750 ± 607	786 ± 82	394 ± 35*
hepatic squalene synthetase	M95591	2931 ± 817	2352 ± 1213	665 ± 358*
kinesin-related protein 2 (KRP2)	U44979	1482 ± 336	1569 ± 56	421 ± 239*

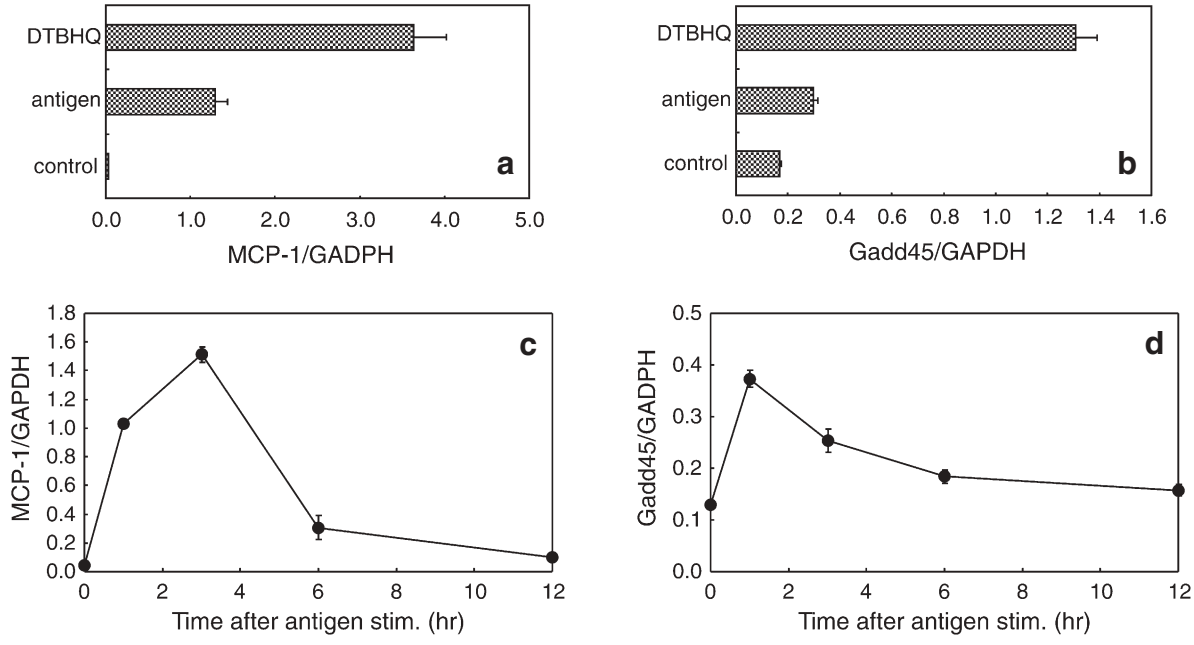

Fig. 2. Real-time PCR for quantification of MCP-1 and GADD45 mRNA. The mRNA was measured by TaqMan PCR as described in Materials and methods. MCP-1 and GADD45 mRNA expression levels are shown as ratios to GAPDH mRNA. MCP-1 (**a**) and GADD45 (**b**) expression in resting RBL-2H3 cells and cells stimulated for 3 h with 10 µg/ml antigen and 10 µM DTBHQ are shown. The kinetic changes in MCP-1 mRNA (**c**) and GADD45 (**d**) mRNA in RBL-2H3 cells after stimulation with 10 µg/ml antigen for 0, 1, 3, 6, and 12 h are also shown.

2a and 2b), and significantly increased to 1.652 ± 0.038 and 0.276 ± 0.015, respectively, after antigen stimulation, and to 3.631 ± 0.387 and 1.310 ± 0.080, respectively after DTBHQ stimulation for 3 h (Fig. 2b and 2c). We also observed that the level of expression of IL-3 was increased from $1.100 \times 10^{-4} \pm 0.049 \times 10^{-4}$ to $3.650 \times 10^{-3} \pm 0.409 \times 10^{-3}$ and $1.462 \times 10^{-1} \pm 0.061 \times 10^{-1}$ after stimulation with antigen and DTBHQ, respectively (data not shown).

When the cells were stimulated with antigen, expression of MCP-1 was increased and peaked within 3 h, while expression of GADD45 peaked at 1 h (Fig. 2c and 2d, respectively). As described previously [22], the DTBHQ-induced change in MCP-1 was much greater, but the pattern was similar to the change in respect to antigen stimulation.

Discussion

Since the rat mucosal mast cell line RBL-2H3 has been widely used as a good model system for IgE-dependent and -independent mast cell activation, the previous data obtained by Chen et al. on global transcripts in RBL-2H3 mast cells by the SAGE method [7] have been very helpful to mast cell researchers.

We used the GeneChip to analyze gene expression profiles in RBL-2H3 cells and found that many genes were up- or down-regulated following stimulation with antigen or DTBHQ. To compare our data on gene expression profiles in RBL-2H3 cells and the data by Chen et al., we set the time interval 3 h after stimulation. And we have already reported that the expression of MCP-1 peaked at 3 h after DTBHQ stimulation [22].

As for mast cells, Saito et al. has previously reported that major basic protein (MBP), which has been known as an eosinophil specific marker, was expressed in human mast cells derived from umbilical cord blood cells [15]. However, in the present study, we could not find out MBP in RBL-2H3 cells, perhaps because of the difference of species.

In this section, we discuss several genes in mast cells divided into 6 categories according to the biological functions of the gene products as shown in Tables 1 and 2.

Cytokines and hormones

A proinflammatory mediator MCP-1 was one of the genes most up-regulated in the cells after antigen and DTBHQ stimulation. The MCP-1 belongs to CC chemokine family, and is a chemoattractant and activating factor that induces monocytes to infiltrate into inflammatory tissues and that induces basophils to release histamine [24]. The fact that the MCP-1 was the most up-regulated gene in the U34A array reemphasized the importance of MCP-1 in proinflammatory responses.

Expression of IL-3, which acts as multi-colony stimulating factor, was dramatically up-regulated by stimulation with DTBHQ alone. In our preliminary experiment, IL-3 transcription was also increased in the RBL-2H3 cells 1 h after antigen stimulation, but decreased to the basal level within 3 h (data not shown). Although production of IL-3 by murine mast cells in response to antigen and calcium ionophore stimulation has already been reported [27], this is the first report on IL-3 mRNA expression in RBL-2H3 cells.

Many Th2- cytokines, including IL-4, IL-9, and IL-13, were also up-regulated by DTBHQ alone, at 3 h after stimulation. Expression of the mRNA of two of them, IL-9 and IL-13 in RBL-2H3 cells was reported first in this study. Since these cytokines are known to accelerate humoral immunity and to be involved in the classswitch to IgE [28], DTBHQ presumably drives a variety of processes involved in allergic inflammation and tissue remodeling.

Relaxin H1 was also markedly up-regulated after both stimulation, and the up-regulation of it by antigen stimulation has already described by Chen et al. [7]. This insulin-like hormone is expressed in several tissues, and plays an important role in tissue remodeling. A recent study showed that G protein-coupled receptors LGR7 and LGR8 are the receptors for relaxin [29]. As the U34A arrays do not contain probes for the LGRs, it is unclear that RBL-2H3 cells express these receptors or not.

Growth arrest, cell death

Expression of GADD45 increased tremendously in both the antigen- and DTBHQ-stimulated RBL-2H3 cells. GADD45 is known to be inducible by stress signals such as UV radiation, X-ray irradiation, DNA alkylating reagents, and oxidative stress, via the p53-dependent pathway in a variety of mammalian cells [25, 26]. GADD45 is involved in cell cycle arrest [30], DNA repair in cooperation with PCNA/cyclin (proliferating cell nuclear antigen) [31], genome stability [32] and signal transduction by stress-responsive MTK1/MEKK4 MAPKKK [33].

Another stress-inducible GADD gene, GADD153, was significantly up-regulated by DTBHQ. Although up-regulation of the GADD gene was reported in 3T3 fibroblasts exposed to the Ca^{2+} pump inhibitor thapsigargin, which has almost the same biological actions as DTBHQ [34], this is the first report on GADD gene induction in mast cells.

When stimulated with DTBHQ, the RBL-2H3 cells showed increased expression of Fas antigen ligand (FasL), which induces apoptotic cell death on Fas-bearing target cells. A previous study showed that murine mast cells do not contain FasL but expressed it when stimulated with PMA and ionomycin [35]. Murine mast cells have also been shown to express functional Fas antigen [36], but no Fas antigen transcripts were detected in RBL-2H3 cells by GeneChip analysis.

In addition, Ink4 (CDK inhibitor 2b) was significantly up-regulated by DTBHQ stimulation. Ink4 is up-regulated in keratinocytes by TGF-β stimulation, and induced a cell cycle arrest by inhibiting CDK4/6 activity [37]. In this study, whether the expression of Ink4 affects the cell cycle of RBL cells is still unclear, but it is probable that DTBHQ-activated mast cells would be growth-arrested and show a kind of stress-inducible response.

Transcription

Some transcription factors and transcriptional regulators were up-regulated after stimulation. The oncoprotein c-Jun forms a heterodimeric transcription factor with c-Fos, called AP1, that binds to a sequence found in the promoters and enhancers of many genes [38]. c-Jun was significantly up-regulated by both stimuli, though c-Fos expression was unchanged (0.9 and 1.0 fold-changed with antigen and DTBHQ, respectively).

We also found that aryl hydrocarbon receptor (AhR) was selectively up-regulated by DTBHQ stimulation. AhR is also known as a “dioxin receptor” because it binds with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and induces expression of metabolical genes such as CYP 1A1 [37]. However, we could not confirm up-regulation of CYP 1A1 by DTBHQ stimulation.

Not only transcription factors, but also the transcriptional repressor, cAMP responsive element modulator (CREM), was significantly up-regulated. CREM is a CRE-binding

protein (CREB)-related transcriptional repressor that inhibits CREB-dependent transcription in a competitive manner [40]. Besides, the transcription factor GATA-1, that is involved in expression of FcεRI α chain and many cytokines, was significantly down-regulated by DTBHQ stimulation [41]. The down-regulation of these transcription factors could participate in a negative feedback loop to control proinflammatory responses in mast cells.

Signal transduction

We found that eight signal transduction-related genes were significantly up-regulated. One example was cytokine-inducible SH2-containing protein (Cish) [42]. Cish is up-regulated in hematopoietic cells by cytokine stimulation and represses cytokine receptor-mediated signaling [43], but it had never been reported to be up-regulated after FcεRI cross-linking.

On the other hand, we observed the down-regulation of several signal transducers, such as interferon regulatory factor (IRF)-1, A2b adenosine receptor, and LIM kinase (LIMK)-1. The reason is not clear, but these down-regulations may reflect negative feedback to make the cells resistant to new signals.

Others

We found that NKR-P2, a member of C-type lectin family, was significantly up-regulated by antigen and DTBHQ stimulation. NKR-P2 has been considered to be a NK cell specific marker [44].

Oxidized LDL receptor was up-regulated by both stimuli. Since this scavenger receptor is ubiquitously expressed and is known to be involved in the uptake of mast cell granule remnants [45], it is likely that mast cells themselves take up their proteoglycan remnants through their own receptors.

We found that progression elevated gene 3 (PEG-3) was significantly up-regulated by both stimulation. The PEG-3 has a 73% nucleotide similarity with the GADD34 gene, but it does not suppress cell cycle unlike GADD genes [46].

We also observed that hexokinase II (HKII) was up-regulated by DTBHQ stimulation. The HKII plays an important role in glycolysis, and is highly expressed in tumor cells [47]. A recent study showed that the overexpression of HKII prevented a proapoptotic protein (Bax)-induced cell death in human embryonic kidney cells [48]. Therefore, HKII may be a potential factor that prevents mast cells from apoptosis induced by an intense stress.

In conclusion, (1) we analyzed expression of thousands of genes in antigen- and DTBHQ-stimulated RBL-2H3 mast cells by GeneChip analysis. (2) We found Ca^{2+}-dependent changes in the expression of several genes that have not been previously reported. (3) Expression of several cytokine genes (MCP-1, IL-3, IL-9, IL-13) and the stress-responsive gene GADD45 were significantly up-regulated. (4) DTBHQ seems to induce proinflammatory responses by stimulating the production of several cytokines through the expression of several transcription factors. (5) The changes in gene expression profile induced by Ca^{2+}-dependent signaling pathway and by FcεRI cross-linking in mast cells were almost the same, but many more stress-inducible genes were up-regulated by the former.

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