Inflammatory response of esophageal epithelium in combined-type esophagitis in rats: A transcriptome analysis

YUJI NAITO[1], MASAAKI KURODA[2], KAZUHIKO UCHIYAMA[2], KATSURA MIZUSHIMA[2],
SATOMI AKAGIRI[2], TOMOHISA TAKAGI[3], OSAMU HANDA[3], SATOSHI KOKURA[3],
NORIMASA YOSHIDA[4], HIROSHI ICHIKAWA[5] and TOSHIKAZU YOSHIKAWA[1,2,3]

[1]Department of Medical Proteomics, [2]Inflammation and Immunology, [3]Department of Biomedical Safety Science,
and [4]Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural
University of Medicine, Kyoto 602-8566; [5]Department of Food Sciences and Nutritional Health,
The Faculty of Human Environment, Kyoto Prefectural University, Kyoto 606-8522, Japan

Abstract. Recent studies have shown that esophageal mucosal inflammatory response is involved in the pathophysiology of gastro-esophageal reflux disease. The aim of the present study was to identify specific gene expression profiles of the esophageal mucosa in a rat model of combined-type chronic reflux esophagitis. Esophagogastroduodenal anastomosis was carried out in male Wistar rats by anastomosing the jejunum to the gastroesophageal junction under diethyl-ether inhalation anesthesia. Esophageal epithelial cells were obtained from esophagi of rats by laser capture microdissection. Preparation of cRNA and target hybridization were performed according to the Affymetrix GeneChip eukaryotic small sample target labeling assay protocol. The gene expression profile was evaluated by the rat toxicology U34 GeneChip. Array data analysis was carried out using Affymetrix GeneChip operating software, Ingenuity pathway analysis software, and Gene Springs software. A comparison between esophagitis and sham-operated rats 2 weeks after the operation revealed that 368 probes (36%) were significantly affected, i.e. 185 probes were up-regulated, and 183 probes were down-regulated, both at levels of at least 1.5-fold in the esophagitis rats. Ingenuity signal analysis of 207 affected probes revealed the interleukin-6 signaling pathway as the most significantly affected caronical pathway. In addition, the expression of many genes associated with cytokine and transcription factor was enhanced in the esophagitis rats. This transcriptome approach provided insight into genes and putative genetic pathways thought to be affected by stimulation with gastro-duodenal refluxates.

Correspondence to: Dr Yuji Naito, Department of Medical Proteomics, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
E-mail: ynaito@koto.kpu-m.ac.jp

Introduction

The pathophysiology of gastro-esophageal reflux disease (GERD) involves contact of the esophageal epithelium with gastric/duodenal juice in the refluxate. Several studies have shown that esophageal mucosal immune and inflammatory responses, characterized by specific cytokine and chemokine profiles, may determine the diversity of esophageal phenotypes of GERD. Fitzgerald *et al* (1) first reported that reflux eso-phagitis is characterized by an acute inflammatory response with significantly increased levels of the proinflammatory cytokines [interleukin-1ß (IL-1ß), IL-8, and interferon-γ] compared with noninflamed squamous esophagus. Isomoto *et al* (2) have also demonstrated that the presence of intra-epithelial neutrophils and eosinophils, which also indicate reflux esophagitis, is associated with high levels of IL-8 and regulated on activation normal T-cell expressed and pre-sumably secreted (RANTES), respectively, and that the IL-8 levels are significantly decreased after proton pump inhibitor treatment. We have also investigated the relationship between the IL-8/monocyte chemoattractant protein 1 (MCP-1) mRNA expression and endoscopic grading of reflux esophagitis according to the Los Angeles classification (3). The expression of IL-8 mRNA determined by real-time PCR correlates with the endoscopic severity of GERD, and increases in patients with nonerosive reflux disease (NERD) compared to normal subjects. There is no correlation between MCP-1 mRNA expression and endoscopic severity, or between the severity of subjective symptoms (QUEST score) and endoscopic grading. These data indicate that chemokine production locally in the esophageal mucosa may be involved in the development and progression of reflux esophagitis, and that gastric acid may play a role in the induction of esophageal inflammation.

Immunohistochemical staining has provided evidence that esophageal epithelial cells produce IL-8 protein (1,3). In order to confirm the epithelial cells as a potential source of cytokines and to identify the molecular mechanism involved in IL-8 production, we have determined whether cultured human esophageal epithelial cells (HEEC) produce IL-8 (4). Stimul-ation of HEEC with cholic acid or taurochenodeoxycholic

acid results in IL-8 production via p38 mitogen-activated protein kinase (MAPK) phosphorylation-dependent pathway. However, the precise mechanism of inflammatory response in esophageal epithelium, especially *in vivo*, has not been fully determined. Some studies of reflux esophagitis have been based on analysis of the expression of a single molecule, or of a relatively limited number of these molecules in esophageal mucosa. DNA microarray techniques have become available that have enabled simultaneous characterization of the mRNA expression pattern of a large number of genes. In the present study, we identified specific gene expression profiles of esophageal epithelial cells in experimental esophagitis, which was recently created by esophagogastroduodenal anastomosis in rats as a model of combined-type chronic reflux esophagitis (5).

Materials and methods

Reagents. All chemicals were prepared immediately before use. An RNeasy Mini kit was purchased from Qiagen (Valencia, CA) and Rat Toxicology GeneChip U34 array and Eukaryotic Small Sample Target Labeling Assay kit were from Affymetrix (Santa Clara, CA). All other chemicals used were of reagent grade.

Rat model of gastroduodenal reflux. Seven-week-old male Wistar rats (Nihon SLC, Hamamatsu, Japan) weighing 200-250 g were used. The rats were housed two per cage under standard laboratory conditions (room temperature, 22±2°C; relative humidity, 55±5%; and a 12-h light/dark cycle). All procedures were prospectively approved by the animal care and use committee of Kyoto Prefectural University of Medicine. Animals were acclimatized in the facility for 1 week before the experiment. Prior to surgery, animals were fasted for 24 h. General anesthesia was induced by diethyl-ether inhalation. A side-to-side esophagogastroduodenostomy was created to induce mixed gastroduodenal reflux according to the previous report (5). At first the transitional region between the fore-stomach and the glandular portion was ligated with a 1-0 silk thread. A longitudinal incision of approximately 7 mm in length was then made on the antimesenteric border of the duodenum. A second incision was made longitudinally along the lower esophagus anteriorly extending 7 mm distally into the proximal stomach. A side-to-side anastomosis was fashioned between the two openings using 7 polypropylene 7-0 sutures. The abdominal wall was closed in two layers with 1-0 silk sutures. Animals were fed only water for 48 h after surgery.

Laser capture microdissection, cRNA amplification, and GeneChip hybridization. We used laser-assisted micro-dissection to obtain cell-specific RNA. Esophageal epithelial cells were identified on cryostat sections (8 μm) of specimens obtained from the esophagus of the rat, and the cells were isolated by laser-assisted microdissection using an LM200 system (Olympus, Tokyo, Japan). A sample containing 200 cells was collected from each esophagus. Although laser capture microdissection can be used to produce cell-specific RNA, this method is limited by the amount of RNA that can be realistically obtained from captured populations of cells, making it likely that the yield will be insufficient for the

commonly used GeneChip assay. To overcome this obstacle, our experiments were performed according to the Affymetrix GeneChip eukaryotic small sample target labeling assay protocol (version II). According to our protocol (6,7), we succeeded in obtaining a sufficient amount of biotinylated cRNA to perform GeneChip analysis from the small amount of esophageal epithelial cells obtained by laser-captured microdissection.

The total RNA was extracted using a Qiagen RNeasy kit and treated with DNase to remove any residual genomic DNA. Briefly, for the first-strand cDNA synthesis, the total RNA sample (1 μl), mixed with T7-Oligo(dT) promoter primer (5 μM, 1 μl), was incubated at 70°C in a thermal cycler for 6 min, cooled to 4°C for 2 min, and then reverse-transcribed for 1 h at 42°C with 3 μl of the RT Premix 1. The sample was then heated at 70°C for 10 min to inactivate the SuperScript II, and was cooled to 4°C. Second-strand cDNA synthesis was carried out by adding 32.5 μl of SS Premix 1 and incubating the sample for 2 h at 16°C. The resulting cDNA was treated with 1 μl T4 DNA polymerase (5 U/μl) for 10 min at 16°C, and was cleaned by ethanol precipitation. After the *in vitro* transcription, the first-cycle cRNA was cleaned using the RNeasy Mini protocol for RNA cleanup from the handbook accompanying the RNeasy Mini kit for cRNA purification (Qiagen). For the second cycle of amplification and labeling, the cRNA sample was mixed with random primers (0.2 μg/μl), incubated at 70°C for 10 min, cooled on ice for 2 min, and incubated at 42°C for 1 h with 5 μl of the RT Premix 2. Second-strand cDNA synthesis was carried out by mixing the sample with 5 μM T7-Oligo(dT) promoter primer, incubating the resulting mixture at 70°C for 6 min, cooling it at 4°C, and re-incubating the sample with 62 μl of SS Premix 2. The resulting cDNA was treated with 1 μl T4 DNA polymerase (5 U/μl) for 10 min at 16°C, and cleaned up by ethanol precipitation. To perform *in vitro* transcription and labeling with the ENZO BioArray high yield RNA transcript labeling kit, the dried double-stranded cDNA pellet was incubated at 37°C for 4 h with 40 μl of the following reagents: 22 μl DEPC-treated water, 4 μl 10X HY reaction buffer, 4 μl 10X biotin labeled ribonucleotides, 4 μl 10X DTT, 4 μl 10X RNase inhibition mix, and 2 μl 20X T7 RNA polymerase. Labeled cRNA target was cleaned up using RNeasy columns. The fragmentation, hybridization, washing, and staining were carried out according to the instructions described in the GeneChip expression analysis technical manual. Affymetrix GeneChip arrays were hybridized with the biotinylated products (5 μg/chip) for 16 h at 45°C using the manufacturer's hybrid-ization buffer. After washing the arrays, hybridized RNA was detected by staining the sample with streptavidin-phycoerythrin. The DNA chips were scanned using a specially designed confocal scanner (GeneChip Scanner 3000, Affymetrix).

Data analysis. Array data analysis was carried out using Affymetrix GeneChip operating software (GCOS) version 1.0. GCOS analyzes image data and computes an intensity value for each probe cell. Briefly, mismatch probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals. To determine the quantitative RNA abundance, the average difference values (i.e., gene expression levels) representing the perfect match-

Table ?. Genes up-regulated (determined as I by GCOS) at least 1.5-fold in esophageal mucosa 2 weeks after esophagogastrostomy (EGJ-stomy).

Accession no.	Gene title	Average difference[a]		Fold change[b]	
		Sham	EGJ-stomy	Log ratio	Ratio
AF048687_s_at	UDP-Gal:ßGlcNAc ß 1,4-galactosyltransferase, polypeptide 6	23.3	301.6	3.4	10.56
rc_AA874919_at	mismatch repair protein	17.6	106.6	3.0	8.00
rc AI045249_at	Transcribed sequence with moderate similarity to protein ref: NP_149107.1 (H. sapiens)	24.9	181.8	2.8	6.96
rc_AI229655_at	Transcribed sequences	96.1	432.3	2.3	4.92
X60767mRNA_s_at	cell division cycle 2 homolog A (S. pombe)	27.2	136.0	2.3	4.92
AF025670_g_at	caspase 6	67.8	140.5	2.0	4.00
U39207_at	cytochrome P450 4F5	50.6	162.6	1.9	3.73
rc_AA875594_s_at	FK506-binding protein 1a	44.6	135.1	1.8	3.48
X96394_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	50.7	149.6	1.7	3.25
rc_AA926129_at	Transcribed sequence with strong similarity to protein ref: NP_446139.1 (R. norvegicus)	39.5	145.9	1.6	3.03
K01721mRNA_s_at	cytochrome P450,2b19	46.5	178.2	1.5	2.83
D85035_g_at	dihydropyrimidine dehydrogenase	92.5	258.9	1.4	2.64
AF003523_s_at	bcl-2 associated death agonist	39.2	87.3	1.3	2.46
U53922_at	DnaJ-like protein	376.4	1068.2	1.2	2.30
AFFX_Rat_beta-actin_3_st		106.8	246.3	1.1	2.14
rc_AI012589_s_at	glutathione-S-transferase, pi 1	356.9	850.2	1.1	2.14
X13933_s_at	Calmodulin 1 (phosphorylase kinase, δ)	236.8	497.8	1.1	2.14
J04791_s_at	ornithine decarboxylase 1	14.2	50.9	1.0	2.00
L26267_at	nuclear factor κ B p105 subunit	218.2	466.7	1.0	2.00
M14050_s_at	heat shock 70k0 protein 5	390.9	837.3	1.0	2.00
M61875_s_at	CD44 antigen	243.3	559.7	1.0	2.00
rc_AA799889_at	Transcribed sequences	103.6	197.5	1.0	2.00
D84450_at	ATPase, Na$^+$/K$^+$ transporting, ß 3 polypeptide	921.0	2215.3	0.9	1.87
rc_AA818226_s_at	cytochrome c oxidase, subunit 4a	2699.6	5211.6	0.9	1.87
rc_AI231354_g_at	stress activated protein kinase α II	154.2	313.3	0.9	1.87
Z78279_g_at	collagen, type 1, α 1	597.9	1050.2	0.9	1.87
M28255_s_at	cytochrome c oxidase, subunit Villa	1900.6	3482.9	0.8	1.74
rc_AI177256_at	Transcribed sequences	134.1	166.9	0.8	1.74
U49930_at	caspase 3	47.8	73.1	0.8	1.74
J02810mRNA_s_at	glutathione S-transferase, mu 1	2511.9	3808.3	0.7	1.62
J05132_s_at	UDP glycosyltransferase 1 family, polypeptide A6	2670.0	4402.5	0.7	1.62
rc_AA955983_at	Transcribed sequence with weak similarity to protein sp: P20291 (R. norvegicus)	726.5	1176.8	0.7	1.62
rc_AI176170_at	FK506-binding protein 1a	594.9	841.5	0.7	1.62
U47315_s_at	brain protein 44-like	816.6	1563.4	0.7	1.62
U47316_s_at	Transcribed sequence with strong similarity to protein ref: NP_038746.1 (M. musculus)	327.4	471.5	0.7	1.62
X54793_at	heat shock protein 60 (liver)	1422.7	2552.6	0.7	1.62
X60328_at	cytosolic epoxide hydrolase	13.2	63.9	0.7	1.62
D00569_g_at	2,4-dienoyl CoA reductase 1, mitochondrial	88.4	127.0	0.6	1.52
D16554_at	polyubiquitin	4334.8	6691.0	0.6	1.52
K00750exon#2-3_at		142.2	219.4	0.6	1.52
K00750exon#2-3_g_at		976.9	1866.2	0.6	1.52
M24604_at	Proliferating cell nuclear antigen	721.4	997.2	0.6	1.52
M76767_s_at	fatty acid synthase	214.4	346.8	0.6	1.52
rc_AA850781_at	Transcribed sequence with strong similarity to protein ref: NP_080628.1 (M. musculus)	45.3	65.9	0.6	1.52
rc_AA875327_g_at	Transcribed sequence with weak similarity to protein ref: NP_072143.1 (R. norvegicus)	425.5	564.4	0.6	1.52
rc_AA893185_at	Transcribed sequence with moderate similarity to protein ref: NP_002483.1 (H. sapiens)	364.7	556.2	0.6	1.52
rc_AA899854_at	topoisomerase (DNA) 2 α	392.4	592.1	0.6	1.52
rc_AI007614_at	Transcribed sequence with strong similarity to protein ref: NP_032722.1 (M. musculus)	352.4	517.5	0.6	1.52
rc_AI104520_s_at	cytochrome c oxidase, subunit VIa, polypeptide 1	951.6	1738.6	0.6	1.52
rc_AI171355_s_at		1160.1	1907.7	0.6	1.52
U77933_at	caspase 2	367.4	623.8	0.6	1.52

[a]Average difference indicates the level of expression of the gene. [b]Fold changes in average difference values were calculated using an Affymetrix software algorithm (GCOS ver. 1.0).

Table II. Genes down-regulated (determined as D by GCOS) at least 1.5-fold in esophageal mucosa 2 weeks after esophago-gastrojejunostomy (EGJ-stomy).

Accession no.	Gene title	Average difference[a]		Fold change[b]	
		Sham	EGJ-stomy	Log ratio	Ratio
J02612mRNA_s_at	UDP glycosyltransferase 1 family, polypeptide A6	672.9	293.2	-0.6	0.66
M55534mRNA_s_at	crystallin, α B	979.2	538.0	-0.6	0.66
rc_AI179916_at	Transcribed sequence with strong similarity to protein ref: NP_057180.1 (H. sapiens)	216.2	125.9	-0.6	0.66
AFFX-BioB-5_at		552.5	310.4	-0.7	0.62
U15211_at	Retinoic acid receptor, α	3451.8	1991.1	-0.7	0.62
U17697 s at	cytochrome P450, subfamily 51	567.9	281.7	-0.7	0.62
AF017393_at	cytochrome P450, subfamily 2F, polypeptide 1	3117.4	1902.1	-0.8	0.57
AFFX-BioB-3_at		488.1	243.8	-0.8	0.57
L17127_g_at	proteasome (prosome, macropain) subunit, ß type 4	692.2	378.3	-0.8	0.57
AFFX-BioC-5_at		2013.3	1090.6	-0.9	0.54
D87336_g_at		2395.0	1070.5	-0.9	0.54
AFFX-BioDn-5_st		414.7	147.9	-1.0	0.50
AFFX-BioDn-3_at		11897.4	5383.7	-1.1	0.47
AFFX-CreX-3_at		25149.0	10238.9	-1.1	0.47
AFFX-CreX-3_st		679.2	230.8	-1.1	0.47
rc_AI169265_at	Transcribed sequence with strong similarity to protein ref: NP_076320.1 (M. musculus)	1990.1	702.2	-1.1	0.47
AFFX-BioC-3_at		1532.3	631.5	-1.2	0.44
D87336_at		705.4	277.2	-1.2	0.44
L32601_s_at	20 α-hydroxysteroid dehydrogenase	385.1	170.6	-1.2	0.44
rc_AI231354_at	stress activated protein kinase α II	137.9	46.1	-1.2	0.44
AFFX-CreX-5_st		571.1	218.8	-1.3	0.41
E03344cds_s_at	peroxisomal membrane protein 3	9.3	2.2	-1.4	0.38
L03294_at	Lipoprotein lipase	288.0	112.4	-1.4	0.32
AFFX-BioDn-5_at		1501.0	546.9	-1.5	0.35
AFFX-CreX-5_at		15387.0	5233.1	-1.5	0.35
L03294_g_at	Lipoprotein lipase	687.8	338.3	-1.5	0.35
rc_AA945054_s_at	cytochrome b5	1326.9	178.2	-2.5	0.18
X79081mRNA_f_at	Cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)	553.5	42.6	-2.7	0.15
J05156_s_at	cytochrome P450, subfamily 11A	117.1	15.0	-2.8	0.14
J02585_at	stearoyl-Coenzyme A desaturase 1	205.7	17.2	-3.4	0.09
rc_AI178204_at	Transcribed sequences	87.9	3.4	-4.2	0.05

[a]Average difference indicates the level of expression of the gene. [b]Fold changes in average difference values were calculated using an Affymetrix software algorithm (GCOS ver. 1.0).

mismatch for each gene-specific probe family were calculated, and the fold changes in average difference values were determined according to Affymetrix algorithms and procedures as follows: I, increase; MI, marginal increase; D, decrease; MD, marginal decrease; and NC, no change. For the pathway analysis, gene probe set ID numbers were imported into the Ingenuity pathway analysis software (Ingenuity Systems, Mountain View, CA). The identified genes were mapped to genetic networks available in the Ingenuity database and were then ranked by a score. The score is the probability that a collection of genes is equal to or greater than the number in a network that could be achieved by chance alone. A score of 3 indicates that there is a 1/1000 chance (significance =0.001) that the focus genes randomly occur in a network. Therefore, gene sets with scores of 3 or higher have a 99.9% confidence level of not being randomly generated. This score was used as the cut-off for identifying gene networks significantly affected by esophagitis. Using the signal intensity of genes selected using the following keywords: cytokine or transcriptional factor by a soft of NetAffx™ Analysis Center (Affymetrix), hierarchical-clustering analysis was performed

with a GeneSpring software package, version 7.0 (Silicon Genetics, San Carlos, CA).

Results and discussion

In the present study, we used a high-density oligonucleotide microarray technique for the mRNA expression profile of esophageal epithelial cells in order to investigate the inflammatory reaction in a rat model of the combined-type chronic esophagitis. We used the GeneChip of rat toxicology U34 array (Affymetrix), which contained 1,031 probes selected from the UniGene database. Comparison of the expression profiles from sham-operated rats and esophagitis rats enabled us to identify differentially regulated genes associated with esophagitis-induced inflammation. Among the 1,031 probes on this array, there were 368 probes (36%) that showed a >1.5-fold difference in expression between the sham-operated and esophagitis groups 2 weeks after the GDE operation; 185 probes (17.9%) were up-regulated and 183 probes (17.7%) were down-regulated. Tables I and II list the genes known to be up- or down-regulated ≥1.5-fold after the operation as compared to

Caronical pathways significantly affected in the esophageal mucosa.

Pathway	Significance	Genes	
		Up-regulated	Down-regulated
IL-6 signaling	2.18×10^{-6}	COL1A1, I11A, JUN, MAP2K2, MAPK14, NFKB1, STAT3, TNF	CYP19A1, FOS, HSPB1, MAPK9, NRAS, TNFRSF1B
p38 MAPK signaling	7.57×10^{-3}	IL1A, MAPK14, TGFB3, TNF	HSPB1, MAPK12, MYC, TNFRSF1B
IL-2 signaling	1.06×10^{-2}	IL2, IL2RB, JUN, MAP2K2	FOS, NRAS

COL1A1: collagen, type I, α 1; CYP19A1: cytochrome P450, family 19, subfamily A, polypeptide 1; FOS: v-fos FBJ murine osteosarcoma viral oncogene homolog; HSPB1: heat shock 27 kDa protein 1; IL1A: interleukin 1, α; IL2: interleukin 2; IL2RB: interleukin 2 receptor, ß; JUN: v-jun sarcoma virus 17 oncogene homolog (avian); MAP2K2: mitogen-activated protein kinase kinase 2; MAPK9: mitogen-activated protein kinase 9; MAPK12: mitogen-activated protein kinase 12; MAPK14: mitogen-activated protein kinase 14; MYC: v-myc myelocytomatosis viral oncogene homolog (avian); NFKB1: nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (p105); NRAS: neuroblastoma RAS viral (v-ras) oncogene homolog; STAT3: signal transducer and activator of transcription 3; TGFB3: transforming growth factor, ß 3; TNF: tumor necrosis factor (TNF superfamily, member 2); TNFRSF1B: tumor necrosis factor receptor superfamily, member 1 B.
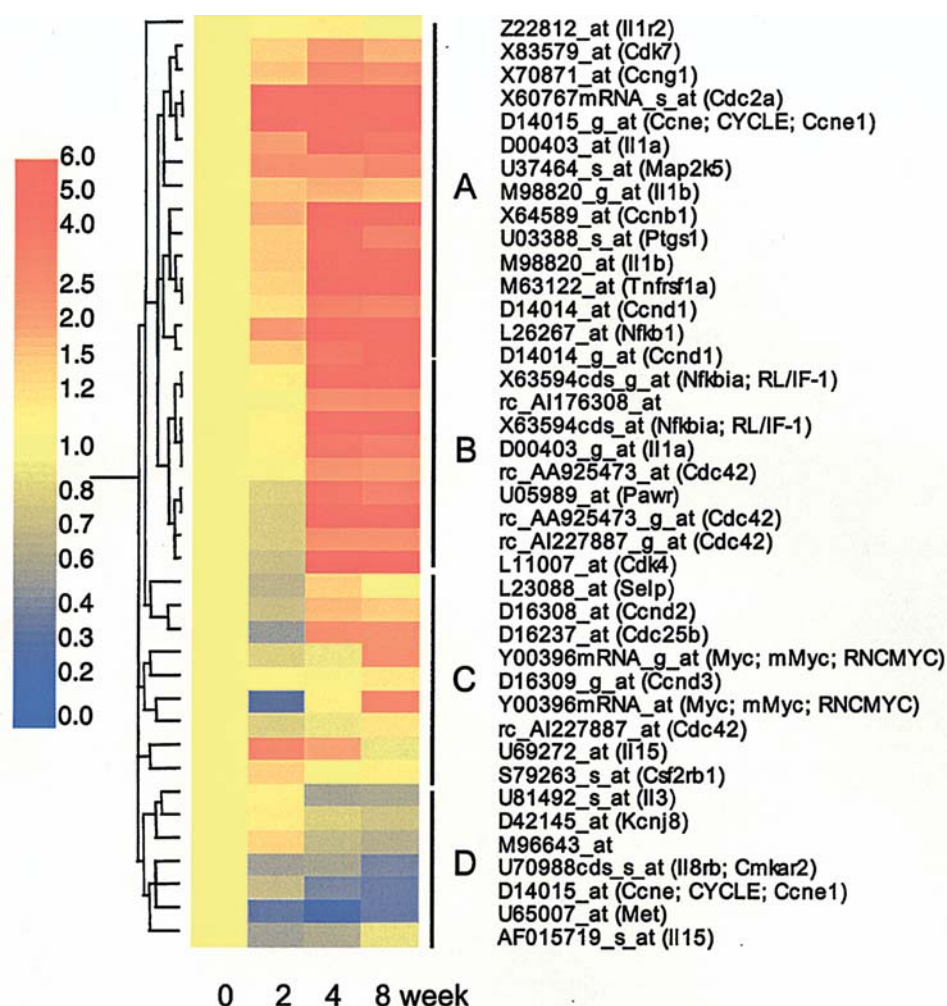

Figure 1. Expression clusters of cytokine-related genes in esophageal mucosa after esophagogastroduodenostomy in rats. In the heirarchical-clustering analysis, a fold-change ratio was calculated using the sham-operated rats and esophagitis samples. Red indicates up-regulated genes, and blue indicates down-regulated genes. Yellow indicates the same expression level as the normoxia sample.

the sham-operated group. Genes that were determined as 'no change' according to Affymetrix algorithms were excluded from Tables I and II. Genes that were up-regulated ≥1.5-fold

were involved in cellular stress responses [heat shock protein 60, 70, and Dna-J, apoptosis-related genes (caspase 2, 3, 6, bcl-2 asscociated death agonist), transcriptional factors (nuclear
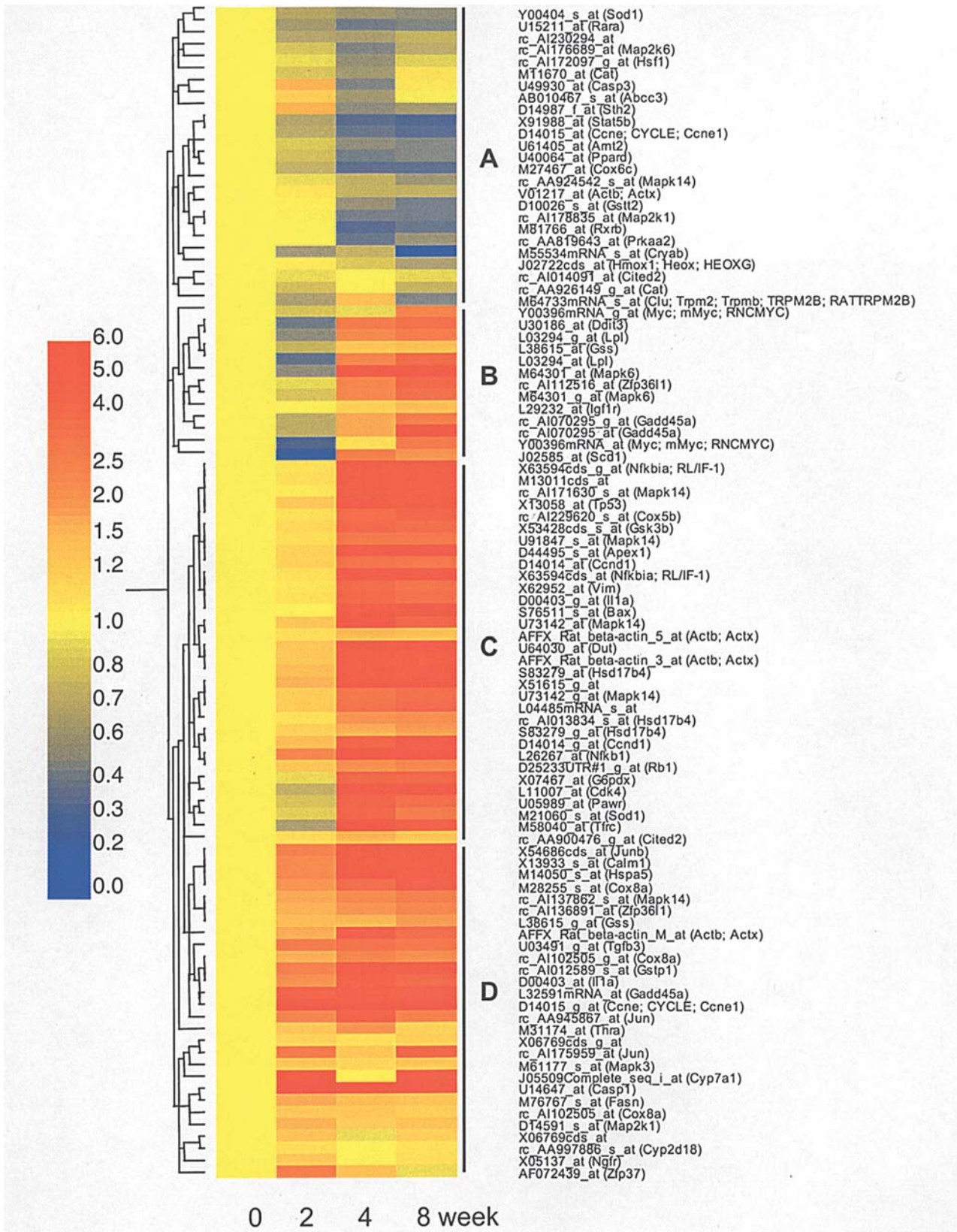
Figure 2. Expression clusters of transcriptional factor-related genes in esophageal mucosa after esophagogastroduodenostomy in rats. In the hierarchical-clustering analysis, a fold-change ratio was calculated using the sham-operated rats and esophagitis samples. Red indicates up-regulated genes, and blue indicates down-regulated genes. Yellow indicates the same expression level as the normoxia sample.

factor κB), DNA damage-associated genes (mismatch repair protein), and enzymes (cytochrome c oxidase, glutathione s-transferase)].

To further refine the list of esophagitis-affected genes, we next investigated which of these genes are known to interact biologically. To this end we carried out pathway analysis on the

3 up- and down-regulated genes using an Ingenuity analysis (IPA) tool. Of these genes, 207 mapped to genetic networks, as defined by the IPA tool. Three caronical networks associated with inflammation were found to be significant in that they had more of the identified genes present than would be expected by chance (Table III): IL-6 signaling, p38 MAPK signaling, and IL-2 signaling. We recently found that the expression of IL-6 mRNA is increased in patients with reflux esophagitis, especially in endoscopic negative reflux disease (unpublished data). In addition, the role of IL-6 in esophagitis was reported by Cheng et al (8), who demonstrated that acid-induced platelet activating factor (PAF) formation induces the production of IL-6 in esophageal mucosa. As IL-6 signaling molecules were most significantly affected in this model of esophagitis, further studies will be necessary to clarify the role of this signaling in the pathogenesis of GERD. In addition, our group recently investigated the role of p38 MAPK in the production of IL-8 by human esophageal epithelial cells (4,9). We found that trypsin or bile acids induce p38 MAPK phosphorylation, determined by Western blotting, and that FR167653, a specific inhibitor of p38 MAPK, significantly inhibits IL-8 production elicited by these stimuli, indicating that trypsin or bile acids induce IL-8 production from esophageal cells via p38 MAPK pathways, which may be involved in the pathogenesis of reflux esophagitis. Further studies will be needed to clarify the role of IL-6, p38 MAPK or IL-2, and especially intracellular signaling, in the combined-type of esophagitis.

In order to focus on inflammation, the expression of genes to be studied was narrowed down to 40 and 102 probes, which were selected using the following keywords: cytokine and transcriptional factor, respectively, using a soft of NetAffx analysis center (Affymetrix). In this study, the expression of these probes at each time point after the GDE operation was shown to be within the limits of 6.0-fold up-regulation (red) or down-regulation (blue) compared to that in the sham-operated group (yellow) using a GeneSpring software. Fig. 1 shows that the probes associated with 'cytokine' were divided into several clusters on the basis of time-kinetics. In group A (Fig. 1), many genes, including the early-response genes (IL-1 and cyclooxygenase), were up-regulated predominantly during the experiment for 8 weeks. In particular, the expression of many probe sets for interleukin (IL-1α, IL-1ß, IL-1 receptor) was markedly up-regulated, and their expression continued or was even further enhanced during the period of esophagitis. In group B, nuclear factor-κB and cell cycle-related genes [cell division cycle 42 homolog (Cdc42) and cyclin-dependent kinase 4 (Cdk4)] were up-regulated at 4 and 8 weeks after the operation. These increases in cell cycle-related genes may result in hyperplasia of the epidermis and basal cells of the esophagus, which are features of esophagitis described in previous reports (5,10,11). The gene expression of group C showed irregular changes during the experiment. Group D included genes such as IL-3 and Met that were down-regulated at least two points compared to the sham.

Fig. 2 shows that the probes associated with 'transcriptional factor' were also divided into several clusters on the basis of time-kinetics. In group A (Fig. 2), many genes were down-regulated at least two points compared to the sham, including CuZn-superoxide dismutase (SOD1), catalase (Cat), and

heme oxygenase-1 (Hmox-1). The down-regulation of these anti-oxidative genes may be associated with aggravation of esophageal injury. Oh et al (10) have demonstrated the increase of malondialdehyde (MDA), an index of lipid peroxidation, and the mucosal depletion of reduced glutathione in the esophagus of reflux esophagitis. Further studies are needed to clarify the role of oxygen radicals and antioxidants in reflux esophagitis. The gene expression of group B was decreased at 2 weeks after the operation and then increased in the late period of the experiment, as did growth arrest and DNA-damage-inducible 45α (Gadd45α) and mitogen-activated protein kinase 6 (Mapk6). Gadd45α is a member of a group of genes induced by agents that damage DNA and/or cause growth arrest. This study showed that three probes of GADD45α (rc_AI070295_at, rc_AI070295_g_at, L32591mRNA_at) in this array were up-regulated after the operation in comparison with the sham-operated rats. Increased GADD45a gene expression has been detected in many mammalian cell types and has been implicated in terminal differentiation (12), growth suppression (13), and apoptosis (14). Previous studies suggested that GADD45 may mediate genotoxic stress-induced apoptosis via activation of c-Jun N-terminal kinase (JNK) and/or p38 MAPK (15,16). These data, including the present information, suggest that enhanced expression of GADD45 followed by MAPK activation may be involved in the apoptosis of eso-phageal epithelial cells after the operation. In groups C and D, many genes, including early- and late-response genes, were up-regulated. Group C (late-response genes) included NF-κB and Mapk14, and group D included v-jun (Jun), heat shock 70 kD protein 5 (Hspa5), glutathione-S-transferase, pi 1 (Gstp1), and several forms of MAPK. This up-regulation of inflammation-associated transcriptional factor may support the hypothesis that esophageal epithelial cells respond by immune and inflammatory pathways after exposure to gastro-duodenal refluxates. In particular, NF-κB activation leads to expression of a large number of NF-κB-dependent genes, which are important mediators of immune and inflammatory responses, including tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor, inducible nitric oxide synthase, intercellular adhesion molecule 1 (ICAM-1), E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and MHC class I and II molecules. The activation of NF-κB has been demonstrated in experimental esophagitis (10), endoscopic negative reflux disease (17), and in Barrett's epithelium and adenocarcinoma (18). These results, including the present data, suggest that the inhibition or regulation of the NF-κB/ proinflammatory cytokine pathway may be an important target for future therapeutic strategies. Detailed clinical studies of the interaction between esophageal epithelium and gastric/duodenal refluxate should make it possible to identify a key therapeutic target molecule that regulates esophageal inflammation.

References

1. Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR and Farthing MJ: Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. Gut 50: 451-459, 2002.
2. Isomoto H, Wang A, Mizuta Y, et al: Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. Am J Gastroenterol 98: 551-556, 2003.

3. Yoshida N, Uchiyama K, Kuroda M, *et al*: Interleukin-8 expression in the esophageal mucosa of patients with gastro-esophageal reflux disease. Scand J Gastroenterol 39: 816-822, 2004.

4. Imamoto E, Yoshida N, Uchiyama K, *et al*: Effects of bile acids and acidic exposure on IL-8 expression in human esophageal epithelial cells. Gastroenterology 124: A-409, 2003.

5. Naito Y, Uchiyama K, Kuroda M, *et al*: Role of pancreatic trypsin in chronic esophagitis induced by gastroduodenal reflux in rats. J Gastroenterol 41: 198-208, 2006.

6. Naito Y, Uchiyama K, Kuroda M, *et al*: Laser capture micro-dissection/GeneChip analysis of gene expression in glomerular cells in diabetic db/db mice. Redox Rep 9: 307-312, 2004.

7. Naito Y, Mizushima K and Yoshikawa T: Global analysis of gene expression in gastric ischemia-reperfusion: a future therapeutic direction for mucosal protective drugs. Dig Dis Sci 50 (suppl 1): S45-S55, 2005.

8. Cheng L, Cao W, Fiocchi C, *et al*: HCl-induced inflammatory mediators in cat esophageal mucosa and inflammatory mediators in esophageal circular muscle in an *in vitro* model of esophagitis increased release of interleukin-6 by oesophageal mucosa in children with reflux oesophagitis. Am J Physiol Gastrointest Liver Physiol 11: 839-843, 2006.

9. Yoshida N, Katada K, Kuroda M, *et al*: Trypsin activates human esophageal epithelial cells to produce inflammatory cytokines through protease-activated receptor 2. Gastroenterology 126: A-501, 2004.

10. Oh TY, Lee JS, Ahn BO, *et al*: Oxidative damages are critical in pathogenesis of reflux esophagitis: implication of antioxidants in its treatment. Free Radic Biol Med 30: 905-915, 2001.

11. Zhang F, Altorki NK, Wu YC, Soslow RA, Subbaramaiah K and Dannenberg AJ: Duodenal reflux induces cyclooxygenase-2 in the esophageal mucosa of rats: evidence for involvement of bile acids. Gastroenterology 121: 1391-1399, 2001.

12. Liebermann DA and Hoffman B: Myeloid differentiation (MyD) primary response genes in hematopoiesis. Blood Cells Mol Dis 31: 213-228, 2003.

13. Jin S, Antinore MJ, Lung FD, *et al*: The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. J Biol Chem 275: 16602-16608, 2000.

14. Sheikh MS, Hollander MC and Fornance AJ Jr: Role of Gadd45 in apoptosis. Biochem Pharmacol 59: 43-45, 2000.

15. Takekawa M and Saito H: A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. Cell 95: 521-530, 1998.

16. Mita H, Tsutsui J, Takekawa M, Witten EA and Saito H: Regulation of MTK1/MEKK4 kinase activity by its N-terminal autoinhibitory domain and GADD45 binding. Mol Cell Biol 22: 4544-4555, 2002.

17. Isomoto H, Nishi Y, Wang A, *et al*: Mucosal concentrations of proinflammatory cytokines and chemokines at gastric cardia: implication of *Helicobacter pylori* infection and gastroesophageal reflux. Am J Gastroenterol 99: 1063-1068, 2004.

18. O'Riordan JM, Abdel-latif MM, Ravi N, *et al*: Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. Am J Gastroenterol 100: 1257-1264, 2005.