Table 2 *Representative highly expressed genes in gastric cancer tissues*

Genes that showed higher expression in cancer tissues than in noncancer tissues ($P < 0.05$) were classified by function, as reported in literature and web database. Seventy-nine representative genes of 162 up-regulated genes are listed below. Ca and N depict the average expression level of each gene in cancer and noncancer tissues, respectively. Cutoff value was set to 80 for Ca and 2.5 for Ca:N (ratio). Accession number denotes GenBank or TIGR database accession number of the probes on the microarray. Note that some genes appear twice due to redundancy of the microarray.

Function	Accession No.	Gene	Symbol	Ca	N	Ca / N	*P* value
Cell adhesion							
	X83228	LI-cadherin	CDH17	197.5	55.9	3.5	4.320E-02
	U40434	Mesothelin	MSLN	119.0	10.0	11.9	2.772E-02
	D21255	OB-cadherin	CDH11	80.6	19.0	4.3	2.072E-03
	M87860	S-lac lectin L-14-II	LGALS2	150.1	12.4	12.1	4.098E-02
	M10321	Von Willebrand factor	VWF	109.1	28.9	3.8	1.394E-02
	X95735	Zyxin	ZYX	292.5	117.7	2.5	3.488E-04
Cell cycle							
	X13293	B-myb	MYBL2	186.5	51.9	3.6	1.896E-05
	S78187	Cdc25B	CDC25B	378.5	133.2	2.8	9.120E-05
	X54941	Cdc28 protein kinase 1	CKS1	251.6	72.1	3.5	1.927E-06
	X54942	Cdc28 protein kinase 2	CKS2	128.2	50.8	2.5	9.120E-05
	U37022	Cyclin-dependent kinase 4	CDK4	199.8	70.4	2.8	1.242E-06
	U41515	Deleted in split-hand/split foot 1	DSS1	432.0	128.3	3.4	6.442E-06
	J04102	Ets 2	ETS2	127.0	10.0	12.7	5.153E-03
	X17644	G1 to S phase transition 1	GSPT1	106.5	26.4	4.0	8.874E-04
	M80359	MAP/microtubule affinity-regulating kinase 3	MARK3	85.5	28.4	3.0	3.663E-03
	D21063	Mitotin	MCM2	128.7	16.6	7.8	1.616E-02
	D21262	Nucleolar and coiled-body phosphoprotein 1	NOLC1	86.6	30.9	2.8	2.652E-05
	M15796	Proliferating cell nuclear antigen	PCNA	170.6	63.4	2.7	9.098E-05
	L76702	Protein phospatase 2A B56-δ	PP2R5D	101.2	18.4	5.5	2.823E-02
Cell motility							
	HT2846	Caldesomn 1	CALD1	101.4	37.5	2.7	6.780E-05
	D83735	Calponin 2	CNN2	286.7	113.7	2.5	8.730E-03
	D45906	LIM domain kinase 2	LIMK2	85.7	10.0	8.6	3.421E-04
	L10678	Profilin 2	PFN2	140.8	22.6	6.2	1.376E-03
	L40379	Thyroid receptor interacting protein 10	TRIP10	178.3	71.3	2.5	1.927E-06
Growth factor related							
	X03363	C-erb-B-2	ERBB2	440.5	68.7	6.4	7.410E-03
	L03840	FGF receptor 4	FGFR4	152.1	13.7	11.1	1.323E-05
	X54489	GRO1oncogene	GRO1	222.4	66.6	3.3	1.869E-02
	D43772	Growth factor receptor-bound 7	GRB7	251.2	10.0	25.1	1.081E-04
	HT3739	Insulin-like growth factor 2	IGF2	655.2	127.7	5.1	2.825E-02
	M94250	Midkine	MK	663.1	197.3	3.4	8.730E-03
	AB000584	Prostate differentiation factor	PLAB	295.3	82.0	3.6	1.691E-03
DNA synthesis							
	D78586	Dihydroorotase and aspartate transcarbamylase	CAD	107.9	41.3	2.6	1.213E-04
	U21090	DNA polymerase δ small subunit	POLD2	120.9	30.4	4.0	1.387E-04
	HT5158	GMP synthetase	GMPS	121.4	29.7	4.1	9.353E-06
	X59543	Ribonucleotide reductase M1 polypeptide	RRM1	97.9	31.7	3.1	4.375E-06
	L16991	Thymidylate kinase	DTYMK	106.3	25.0	4.3	2.090E-04
Chromosome							
	L47276	α topoisomerase truncated-form	TOPATR	198.4	47.5	4.2	2.644E-05
	J04088	DNA topoisomerase II	TOP2A	152.5	45.9	3.3	3.071E-04
	X60486	H4 histone family, member G	H4FG	240.8	66.4	3.6	1.603E-02
	U47077	DNA dependent protein kinase, catalytic subunit	PRKDC	97.0	38.3	2.5	5.018E-05
	M61764	Tubulin, γ polypeptide	TUBG	90.1	14.1	6.4	1.598E-04
Transcription							
	L24203	Ataxia-telangiectasia group D-associated protein	ATDC	105.3	10.0	10.5	4.936E-03
	U18018	E1A enhancer-binding protein, E1A-F	E1A-F	95.3	10.0	9.5	9.229E-06
	L03411	RD-RNA binding protein	RD	129.7	50.4	2.6	2.927E-06
	HT2370	RNA polymerase II, 14.5kD subunit	RPB14.5	192.1	55.6	3.5	9.098E-05
	U51586	SIAH binding protein 1	SIAHBP1	246.0	43.8	5.6	3.667E-05
	X17567	Small nuclear ribonucleoprotein polypeptide B	SNRNPB	1024.0	396.8	2.6	1.592E-05
	L25444	TBP-associated factor TAFII80	TAF2E	125.1	29.5	4.2	3.051E-03
	X70683	SRY (sex determining region Y)-box 4	SOX4	133.9	45.9	2.9	2.239E-05
Angiogenesis							
	Y00787	Interleukin 8	IL8	474.1	113.7	4.2	5.269E-03
	J03040	Osteonectin	SPARC	816.8	209.0	3.9	1.896E-05
	L12350	Thrombospondin 2	THBS2	148.9	23.2	6.4	1.336E-05
Extracellular matrix							
	Z74615	Collagen, type I, α 1	COL1A1	696.9	216.4	3.2	9.353E-06

Table 2 *Continued*

Z74616	Collagen, type I, α 2	COL1A2	867.4	139.4	6.2	2.927E-06
X06700	Collagen, type III, α 1	COL3A1	637.9	228.4	2.8	2.710E-04
M24766	Collagen, type IV, α 2	COL4A2	170.3	10.0	17.0	5.785E-06
X05610	Collagen, type IV, α 2	COL4A2	746.8	252.5	3.0	6.442E-06
M26576	Collagen, type IV, α 1	COL4A1	484.4	135.7	3.6	1.896E-05
M11718	Collagen, type V, α 2	COL5A2	194.5	73.4	2.7	6.256E-03
X52022	Collagen, type VI, α 3	COL6A3	628.4	135.0	4.7	3.667E-05
HT2267	Collagen, type VII, α 1	COL7A1	119.1	46.7	2.6	8.933E-04
L22548	Collagen, type XVIII, α 1	COL18A1	130.1	10.0	13.0	2.707E-06
HT4850	Elastin	ELN	181.4	72.0	2.5	1.394E-02
HT3742	Fibronectin	FN1	521.0	45.2	11.5	3.488E-04
X02761	Fibronectin	FN1	887.3	105.8	8.4	2.652E-05
U20758	Osteopontin	SPP1	177.8	16.8	10.6	4.358E-06
U16306	Versican	CSPG2	196.8	55.1	3.6	1.693E-03
Extracellular matrix remodeling						
X83573	Arylsulfatase E	ARSE	141.4	55.5	2.5	1.197E-02
X82153	Cathepsin K	CTSK	155.1	43.5	3.6	4.449E-04
X54925	Matrix metalloproteinase 1	MMP1	195.1	27.7	7.0	1.525E-03
X05232	Matrix metalloproteinase 3	MMP3	118.5	10.0	11.9	2.951E-04
L22524	Matrix metalloproteinase 7	MMP7	282.6	46.2	6.1	1.693E-03
X57766	Matrix metalloproteinase 11	MMP11	229.1	93.1	2.5	6.262E-03
L23808	Matrix metalloproteinase 12	MMP12	202.8	55.3	3.7	1.193E-02
Z48481	Membrane type-matrix metalloproteinase 1	MMP14	116.0	45.2	2.6	5.018E-05
X02419	Plasminogen activator, urokinase	uPA	188.2	37.9	5.0	6.797E-05
L33799	Procollagen C-endopepidace enhancer	PCOLCE	188.1	10.4	18.2	9.120E-05
L06419	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase	PLOD	178.9	42.6	4.2	9.816E-04
M22612	Protease, serine, 1	TRY1	127.6	14.2	9.0	1.287E-02

pairs in 33 genes, three pairs in 26 genes, two pairs in 17 genes, and one pair in 4 genes, showing a high concordance rate between the data obtained by RT-PCR and data obtained by microarray. Additionally, these genes were classified in terms of function by referring to the literature and web database[4] as shown in Table 2. Supplementary tables are available at Cancer Research Online.[5]

Genes Associated with Lymph Node Metastasis. Advanced gastric cancer often accompanies lymph node metastasis in the course of progression. Some genes such as *IL8, VEGF, OPN, CD44v9,* and *MMP9* are reportedly related to gastric cancer metastasis and invasion in general (13). However, there are few studies that focus on lymph node metastasis. To explore genes associated with this type of metastasis, we compared 15 cancer samples with metastasis to 7 cancer samples without metastasis and subsequently to 8 noncancerous tissues. Nine genes showed a distinct expression pattern exclusively in cancer tissues with lymph node metastasis ($P < 0.05$), a >2-fold change as compared with any of the other groups (Fig. 2*A*). These genes included matrix remodeling genes, such as *FN1* and *PCOLCE,* and *PFN2,* which affects cell motility by regulating actin polymerization (14). Among 9 genes identified, association of *Oct-2* with metastasis was intriguing, because it has been generally regarded not as a gastric but as a lymphoid or neuronal cell-specific transcription factor (15). To investigate which cells are expressing *Oct-2,* immunohistochemical analysis was performed. Strong immunoreactivity was observed in gastric cancer cells with lymph node metastasis and in some infiltrating lymphocytes, but not in cancer cells without metastasis (Fig. 3).

Genes Associated with Histological Types. Gastric cancer is generally classified into two major histological types according to Lauren's classification: intestinal type and diffuse type, which roughly correspond to the highly and poorly differentiated type, respectively (16). Many previous works indicate distinct genetic changes and expression pattern of a subset of genes between these two types. Loss of *E-cadherin* expression and K-*sam* amplification are predominant in diffuse-type cancer, and mutation or nuclear accumulation of *β-catenin* and amplification of c-*erb*B2 are predominant in intestinal-type cancer, whereas mutation or nuclear accumulation of *p53* is frequently observed irrespective of histological type (3, 4, 16). As described in Table 1, immunohistochemical analysis of *E-cadherin, β-catenin,* and *p53* in this study is consistent with the findings of these previous works. Moreover, extremely high expression of c-*erb*B2 in the microarray data, which is suggestive of gene amplification, was observed exclusively in intestinal-type cancer (data not shown). Gastric cancer samples used in the current study are therefore quite adequate for further analysis. To identify novel genes associated with histological types based on transcription analysis, we compared gene expression between the two types. Fifteen genes showed >2-fold differential expression between the two types ($P < 0.05$; Fig. 2*B*). Overexpression of intestinal enzymes *GALC* (17), *GUCY2C* (18), and *GPX2* (19) and reduced expression of gastric protein MSMB (20) in intestinal-type cancer were identified, reflecting intestinal differentiation in intestinal-type gastric cancer. Additionally, *LI-cadherin* (CDH17), one of the cadherin family genes, which have crucial roles in cell-cell adhesion, showed preferential expression in intestinal-type cancer. Because expression of *LI-cadherin* is observed in intestinal cells and hepatocytes (21), but not in gastric epithelium (22), it can also be regarded as one of the intestinal differentiation markers.

DISCUSSION

In this study, we have globally analyzed gene expression of gastric cancer tissues and noncancerous tissues to elucidate characteristic changes associated with carcinogenesis and progression in gastric cancer. Cancer tissues and noncancerous tissues were distinguished by gene expression profiling alone, indicating that array analysis of whole cancer tissues can efficiently detect characteristics of gastric

[4] http://www.ncbi.nlm.nih.gov/LocusLink/.

[5] Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

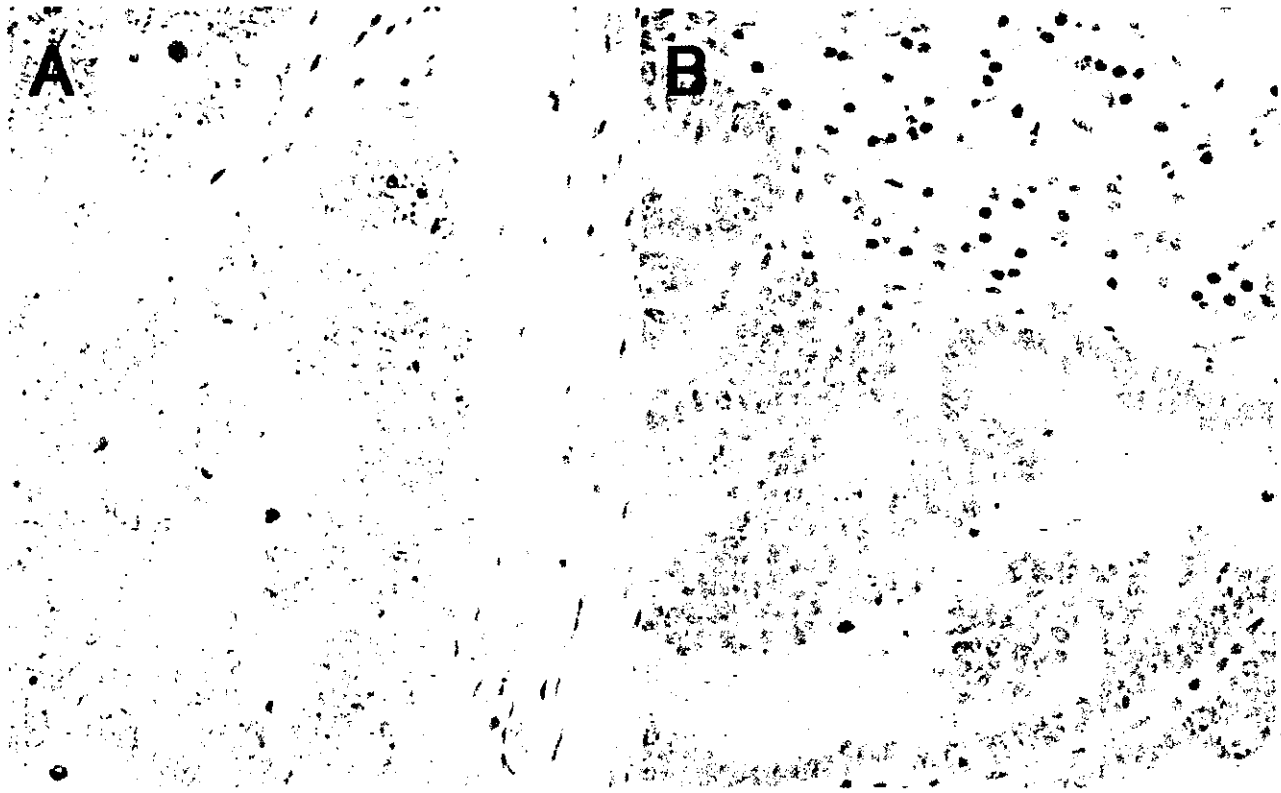

Fig. 3. Immunohistochemical analysis of *Oct-2*. *A*, metastasis-positive cancer tissue (T8). Gastric cancer cells showed strong immunoreactivity. *B*, metastasis-negative cancer tissue (T12). No immunoreactivity was observed for cancer cells. Note that some infiltrating lymphocytes are immunostained and can serve as a positive control. These photomicrographs depict ×400 magnification.

cancer by integrating alteration of gene expression in cancer cells and stromal cells. When we reviewed genes that were highly expressed in gastric cancer tissues (Table 2),[5] we could readily extract two major features: (*a*) high proliferative status of cancer cells; and (*b*) reactive status of stromal cells. Genes classified in the cell cycle, growth factor-related, DNA synthesis, chromosome, and transcription category were related to high proliferative status of cancer cells and expressed predominantly by cancer cells. Some genes have been previously reported to show high expression in gastric cancer, such as *TOP2A*, *CKS1*, *CKS2*, *CDK4*, and *PCNA* (23), *FGFR4* (24), *IGF2* (25), *CDC25B*, *ERBB2* (3), and *GRB7* (26). On the other hand, genes classified in the ECM, ECM remodeling, and angiogenesis category were related to the reactive status of stromal cells and expressed mainly by stromal cells and partly by cancer cells. When we referred to other comprehensive studies on gene expression specific to endothelium and cancer invasion, these genes could be characterized more precisely. Genes expressed predominantly in the endothelium have recently been identified with serial analysis of gene expression (27). Among the genes listed in Table 2,[5] *VWF*, *SPARC*, *COL18A1*, *COL4A2*, and *GEM* were expressed in both normal and tumor endothelium, whereas *CST4*, *THY1*, *MMP11*, *COL1A2*, *COL6A3*, *COL3A1*, and *COL1A1* were expressed exclusively in tumor endothelium. Interestingly, the most abundant six of nine collagen genes were of endothelial origin, highlighting a crucial role of angiogenesis in the formation of desmoplasia, a fibrotic change seen frequently in gastric cancer. Genes related to cancer invasion included most of endothelium-expressed genes mentioned above and cancer-expressed genes *CALD1* (28), *HSPA1A* (29), *NNMT* (30) and *LRP1* (23, 31). Besides, high expression of *MAGE3* (32), *VIL1* (33), and *SOD2G* (34) in gastric cancer has been reported previously. Many other genes identified here were also associated with various types of cancer. For example, high expression of *MSLN* (35) and *KLK6* (36) in ovarian cancer, *GRO1* in malignant melanoma (37), and *H19* (38), *MK* (39), and chaperone genes (40) in many types of cancer have been reported previously.

We identified several genes associated with lymph node metastasis (Fig. 2*A*). *FN1* and *PCOLCE* are genes related to matrix remodeling (41). The involvement of *FN1* in cell migration and metastasis has been well documented (42, 43). *PFN2* affects cell motility by regulating actin polymerization in response to outer signals (14). Growth factor *IGF2* also promotes cell motility (44). It is likely that cell motility enhanced by these genes can lead to metastasis. Unexpectedly, *Oct-2* was highly expressed by cancer cells with lymph node metastasis. *Oct-2* is a POU domain transcription factor that shows a restricted expression pattern in lymphoid cells and neuronal cells and is involved in transcription of immunoglobulin genes in B cells (15, 45). There is only one report of *Oct-2* expression by cancer cells (46); however, constitutive expression *in vitro* of Oct-2 was confirmed by RT-PCR in 7 of 11 gastric cancer cells examined (data not shown), suggesting its frequent ectopic expression by cancer cells. We have previously reported overexpression of MHC class II genes via up-regulation of *CIITA*, a transactivator of MHC class II genes, in a gastric cancer cell line with high metastatic potential to lymph nodes in a nude mouse model (47). It is extremely intriguing that these lymphoid cell-specific genes are associated with lymph node metastasis. It remains to be investigated whether these genes are functionally relevant to lymph node metastasis of gastric cancer.

We further identified genes associated with histological type of

gastric cancer (Fig. 2*B*). Because *GALC* (17), *GUCY2C* (18), and *GPX2* (19) are expressed predominantly in the intestine, overexpression of these genes can be regarded as intestinal differentiation of cancer cells. On the other hand, *MSMB* is predominantly expressed in gastric antrum (20), and its selective down-regulation can be viewed as dedifferentiation from the gastric phenotype. Consistent with the current study, *HSPA1B* (29) and *CDKN2A* (48) have been reported to show differential expression between the two types. Moreover, *LI-cadherin* showed high expression in intestinal-type gastric cancer, which is in line with a recent immunohistochemistry study (49). Because *LI-cadherin* could already be detected in intestinal metaplasia, a cancer-predisposed lesion for intestinal-type gastric cancer (50), the transcriptional regulator of *LI-cadherin* may have crucial roles in the multistep carcinogenesis of intestinal-type gastric cancer.

Advanced gastric cancer is generally refractory to chemotherapy by anticancer drugs, which leads to poor prognosis. Accordingly, targets of gastric cancer therapeutics have been recently extended from molecules of cancer origin to molecules of stroma origin, such as those related to angiogenesis and matrix remodeling (51, 52). Because our study was based on whole tissue samples, the list of genes up-regulated in cancer tissues contained and may still contain many genes for therapeutic target molecules of stroma. Precise prediction of metastases in neighboring lymph nodes remains very difficult but can provide evidence for selecting optimal therapy between surgical and endoscopic resection or optimal extent of lymph node dissection in case of surgery. If examination of the metastasis-associated genes identified in this study were applicable in the future to predict lymph node metastasis from biopsy samples, it would undoubtedly be of great clinical value. In conclusion, the genes described in the current study should therefore provide valuable resources not only for basic studies, such as understanding molecular mechanism of carcinogenesis, progression, and metastasis, but also for clinical applications, such as development of novel diagnostic markers and identification of therapeutic targets in gastric cancer.

ACKNOWLEDGMENTS

We thank Akima Harada and Kaori Shiina for immunohistochemical analysis, Hiroko Meguro for GeneChip analysis, and Saori Fukui and Erio Fujita for excellent technical support.

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Am J Physiol Gastrointest Liver Physiol 282: G338–G348, 2002.
First published October 24, 2001; 10.1152/ajpgi.00376.2001.

Fish oil feeding alters liver gene expressions to defend against PPARα activation and ROS production

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Received 24 August 2001; accepted in final form 19 October 2001

Takahashi, Mayumi, Nobuyo Tsuboyama-Kasaoka, Teruyo Nakatani, Masami Ishii, Shuichi Tsutsumi, Hiroyuki Aburatani, and Osamu Ezaki. Fish oil feeding alters liver gene expressions to defend against PPARα activation and ROS production. *Am J Physiol Gastrointest Liver Physiol* 282: G338–G348, 2002. First published October 24, 2001; 10.1152/ajpgi.00376.2001.—Fish oil rich in n-3 polyunsaturated fatty acids has been shown to reduce the risk of cardiovascular diseases partly by reduction of blood triglyceride concentration. This favorable effect mainly results from the combined effects of inhibition of lipogenesis by decrease of SREBP-1 and stimulation of fatty acid oxidation by activation of peroxisome proliferator-activated receptor-α (PPARα) in liver. However, because fish oil is easily peroxidized to form hydroperoxides and increases oxidative stress, some defense mechanism(s) against oxidative stress might occur. To understand these complex effects of fish oil diet, the gene expression profile of mice liver was analyzed using high-density oligonucleotide arrays. High-fat diet (60% of total energy intake) as either safflower oil or fish oil (tuna) was given to mice. After 6 mo of feeding, expression levels of a total of 6,521 genes were analyzed. In fish oil diet compared with safflower oil diet, immune reaction-related genes, antioxidant genes (several glutathione transferases, uncoupling protein 2, and Mn-superoxide dismutase), and lipid catabolism-related genes upregulated, whereas cholesterol and fatty acid synthesis-related genes and 17-alpha hydroxylase/C17–20 lyase and sulfotransferases related to production of endogenous PPARα ligands and reactive oxygen species (ROS) downregulated markedly. Because upregulation of these antioxidant genes and downregulation of sulfotransferases were also observed in mice administered fenofibrate, altered gene expression related to antioxidant system observed in fish oil feeding was mediated directly and indirectly by PPARα activation. However, downregulation of 17-alpha hydroxylase/C17–20 lyase was not due to PPARα activation. These data indicate that fish oil feeding downregulated the endogenous PPARα-activation system and increased antioxidant gene expressions to protect against ROS excess.

n-3 fatty acids; fibrate; sulfotransferase; glutathione transferase; dehydroepiandrosterone

IN RODENTS, FISH OIL FEEDING showed less obesity and lower blood triglyceride levels relative to other dietary oils (4, 13). In humans, increased intake of fish oil showed protective effects against cardiovascular disease (27), but concern remains that increased intake of fish oil may lead to increased lipid peroxidation (23). Contained in fish oil are n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are taken into the body, are mostly delivered to liver (29), and are easily peroxidized to form hydroperoxides and their secondary degradation products (9). These are considered to be deleterious to tissues (37). Thus liver is the primary target organ for oxidative stress in fish oil feeding. Fish oil administration and fibrate, which are peroxisome proliferator-activated receptor-α (PPARα) activators, manifest substantial increase in expression of H_2O_2-generating peroxisomal fatty acyl CoA oxidase, the first enzyme of the classic peroxisomal fatty acid β-oxidation system, and of microsomal Cyp 4A1 and 4A3 genes (41). Disproportionate increases in H_2O_2-generating enzymes and H_2O_2-degrading enzyme catalase and reductions in glutathione peroxidase activity by peroxisome proliferators lead to increased oxidative stress in liver. However, the results of fish oil feeding to animals showed slight increases in the tissue level of thiobarbituric acid reactive substances (33). Thus fish oil feeding may enhance the hepatic antioxidant defense with several mechanism(s).

To identify genes responsible for metabolic alterations and antioxidant systems of fish oil feeding, the gene expression profile of 6,521 genes using oligonucleotide arrays in high-carbohydrate diet, high-safflower oil diet, and high-fish oil diet-fed mice was examined in mice liver. In addition, to examine whether the altered gene expression of fish oil feeding is via PPARα activation, mRNA levels of liver from mice administered fenofibrate were examined by Northern blotting.

MATERIALS AND METHODS

Diet experiment. Female C57BL/6 mice were obtained from Tokyo Laboratory Animals Science (Tokyo, Japan) at 7 wk of age and fed a normal laboratory diet for 1 wk to stabilize the

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metabolic conditions. Mice were exposed to 12:12-h light-dark cycle and maintained at a constant temperature of 22°C.

For gene-chip analysis, mice were divided into three groups (n = 5–6 in each group). The first group was given a high-carbohydrate diet that, on a calorie basis, contained 63% carbohydrate, 11% fat, and 26% protein. In the high-carbohydrate diet, safflower oil was used as source of fat. The second group was given a high-safflower oil-rich diet containing 14% carbohydrate, 60% safflower oil, and 26% protein. Safflower oil used here was high-oleic type, containing 46% oleic acid (18:1n-9) and 45% linoleic acid (18:2n-6) from total fatty acids. The third group was given a high-fish oil diet containing 14% carbohydrate, 60% fish oil, mainly from tuna, and 26% protein. Fish oil contained 7% EPA (20:5n-3) and 24% DHA (22:6n-3) and was kindly provided by NOF (Tokyo, Japan). In this study, to elucidate the effects of fish oil feeding, we used a very high-fat diet (60% of total energy intake). The materials and methods of preparation and fatty acid composition of diet were the same as those used in our previous studies (15, 38). Mice were fed each diet for 6 mo. At the end of the experiments, animals were anesthetized at about 10:00 AM by intraperitoneal injection of pentobarbital sodium (0.08 mg/g body wt, Nembutal; Abbot, North Chicago, IL). Liver was isolated immediately, weighed, and homogenized in guanidine-thiocyanate, and RNA was prepared by the method described by Chirgwin et al. (3). RNAs were used for gene-chip analysis and Northern blotting.

To confirm the results of gene-chip analysis and to examine whether up- and downregulation of these genes by 6 mo fish oil feeding were also observed in a short-term feeding period and were due to activation of PPARα, Northern blots from the four groups of mice were made. Three groups were the same used in gene-chip analysis, and the fourth group was treated with a direct PPARα ligand fenofibrate (Sigma, St. Louis, MO) mixed in high-carbohydrate diet. Because mouse consumed ~1.5–2.0 g chow/day, doses of 0.5% (wt/wt) mixed in diet correspond to 410–550 mg·kg body $wt^{-1}\cdot day^{-1}$. Mice were fed each diet for 1 wk and killed similar to gene-chip experiments to obtain RNA. We chose a 1-wk feeding period as short-term feeding, because upregulation of target genes of PPARα such as acyl-CoA synthetase (ACS) and lipoprotein lipase (LPL) were observed in a 1-wk period of fish oil feeding or fenofibrate administration (data not shown).

Gene-chip analysis of gene expression. Poly $(A)^+$ RNA was prepared from pooled total RNA from five animals of each group by using an mRNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ). Double-stranded cDNA (Superscript Choice System, GIBCO BRL) was made from 1 μg of mRNA with a T7-$(dT)_{24}$ primer containing T7 RNA polymerase promoter site (Amersham Pharmacia). Biotinylated complementary RNA was made from 1 μg of cDNA using BioArray high-yield RNA transcript labeling kit (Enzo) and then fragmented to ~100–200 nucleotides. Ten micrograms of these in vitro transcripts were hybridized to Affymetrix Mu 6500 microarray for 16 h at 45°C with constant rotation at 60 rpm (21). Chips were washed and stained with streptavidin-phycoerythrin (10 μg/ml, Molecular Probes) and biotinylated goat antistreptavidin (3 μg/ml, Vector Laboratories) using tEukGE-WS2 protocol on Affymetrix fluidics station. Chips were scanned using Hewlett-Packard confocal laser scanner and visualized using Affymetrix Gene Chip 3.1 software (Affymetrix). Fluorescence intensity from the safflower oil- and fish oil-fed groups was normalized to that from the carbohydrate-fed group by equating the overall fluorescence intensity for the entire chip of each group. The average values were scaled to 100 so that all chips could be directly compared, and the data were imported into File Maker Pro (File Maker).

In order of fold-change levels in gene expression in liver from fish oil-fed mice relative to safflower oil-fed mice, genes increased more than twofold and decreased less than twofold are listed and examined. Difference call, which defines genes increased or decreased, is derived from this software and is classified in five stages: transcript has increased, decreased, marginally increased, marginally decreased, or has exhibited no change in expression level. Fold-change calculation was carried out as an indication of the relative change of each transcript represented on the probe array. Differentially expressed genes were identified using the following criteria. Absolute call is present, and average difference was >150. Absolute call, which was calculated by this software using several markers, is an indicator of the presence or absence of each gene transcript. The average difference value is a marker of abundance of each gene obtained by comparing the intensity of hybridization to 20 sets of perfectly matched 25-mer oligonucleotides relative to 20 sets of mismatched oligonucleotides using Affymetrix Gene Chip 3.1 software.

Preparation of cDNA probe for Northern blot. The cDNA fragments for cytochrome *P*-450 17-alpha hydroxylase/c17–20 lyase, sulfotransferases, kappa-immunoglobulin (constant region), gelsolin, and glutathione transferase theta-class type 2 were obtained by PCR from first strand cDNA using mouse liver total RNA. First strand cDNA was prepared using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) primed with oligo(dT). The PCR primers used were as follows: cytochrome *P*-450 17-alpha hydroxylase/c 17–20 lyase, 5′ primer, 5′-CTACACCTGGCTGCCATGT-3′, and 3′ primer, 5′-GCCTGATACGAAGCACTTCT-3′; hydroxysteroid sulfotransferase, 5′ primer, 5′-AGGAACGAACTGGCTGAATG-3′, and 3′ primer, 5′-CTTGGGCTACTGTGAAGTGA-3′; phenol/aryl form sulfotransferase, 5′ primer, 5′-CCACATTGCAAAGCCTACAC-3′, and 3′ primer, 5′-CATTAGCTTGGCATAGTGGG-3′; kappa-immunoglobulin (constant region), 5′ primer, 5′-GATGCTGCACCAACTGTATC-3′, and 3′ primer, 5′-AACAGTGGTAGGTCGCTTGT-3′; gelsolin, 5′ primer, 5′-GCACTATGGTGGTGGAGCA-3′, and 3′ primer, 5′-CGTTGGCAATGTGGCTGGA-3′; glutathione transferase theta-class type 2 primer, 5′-GCTTGCTGTTATCGAACGCA-3′, and 3′ primer, 5′-TGGTCAGACCACTCAAGGAA-3′. PCR was performed with *Taq* DNA polymerase (Takara, Shiga, Japan). Amplification was made by using the following program: *segment 1*: 94°C, 1 min; *segment 2*: 30 cycles of 98°C for 20 s, 68°C for 2 min; and *segment 3*: 72°C for 10 min. The amplified products were subcloned into pGEM-T Easy vector (Promega, Madison, WI) and verified by sequencing. The cDNA probes for rat glutathione transferase Ya subunit were kindly provided by Dr. Nakagawa at Kitasato University, and rat Mn-SOD was provided by Dr. Ookawara at Hyogo Medical University. These cDNA were used as probes for Northern blotting.

Northern blotting. A portion of RNA (15 μg/lane) was denatured with glyoxal and dimethyl sulfoxide and analyzed by electrophoresis in 1% agarose gels. After transfer to Nylon membranes (New England Nuclear, Boston, MA) and ultraviolet cross-linking, RNA blots were stained with methylene blue to locate 28S and 18S rRNAs and to ascertain the amount of loaded RNAs (35). The blots were hybridized overnight at 42°C with cDNAs that had been labeled with [^{32}P]dCTP (New England Nuclear) by a random prime labeling kit (Amersham Pharmacia Biotech). The filters were washed several times with 1× SSC, 0.1% SDS at room

temperature, washed twice at 50°C, and then exposed to X-ray film at −80°C.

Statistics. Statistical comparisons of the groups were made by one-way ANOVA, and when they were significant, each group was compared with the others by Fisher's protected least-significant differences test (Statview 5.0 Abacus Concept, Berkeley, CA). Statistical significance is defined as $P < 0.05$. Values are means ± SE.

RESULTS

Phenotypic comparison of mice fed three different diets for 6 mo. The difference of body and tissue weights, blood lipid concentrations, and other metabolic parameters after 5–6 mo feeding are described in our previous studies (13, 15, 38). Briefly, compared with carbohydrate diet, safflower oil diet resulted in two- to threefold increase of wet parametrial white adipose tissue weight with concomitant 40% increase of body weight, but fish oil feeding did not increase white adipose tissue and body weight. The average energy intake of mice fed each diet was not significantly different; energy intakes of mice fed carbohydrate diet, safflower oil diet, and fish oil diet were 7.4 ± 0.5, 7.7 ± 0.9, 7.9 ± 0.5 kcal·day^{-1}·mouse^{-1}, respectively, when they were measured at 12–13 wk feeding. Liver weight from fish oil-fed mice was 60% greater than that from carbohydrate-fed mice, possibly through peroxisomal proliferation. Fish oil feeding decreased liver triglyceride and cholesterol concentration by 62% and 35%, respectively, compared with safflower oil feeding.

Altered gene numbers among three different diets. Table 1 shows the number of liver genes whose expression levels were altered after a 6-mo feeding period. Of the 6,521 genes, including 3′ expressed sequence tags (ESTs), analyzed, 1,669; 1,925; and 1,928 were expressed at significant levels (absolute call is present), and 975; 1,039; and 992 were expressed at substantial levels (absolute call is present, and average difference is >150) in carbohydrate-, safflower oil-, and fish oil-fed female mice, respectively. Of these, in safflower oil- and fish oil-fed mice, compared with carbohydrate-fed mice, 132 (13%), and 117 (12%) genes, respectively, significantly and substantially upregulated (difference call is increased, and its average difference is >150), whereas those of 14 (1%) and 48 (5%) genes, respectively, downregulated significantly (difference call is decreased, and average difference of carbohydrate-fed mice is >150). Within fat diet-fed groups, numbers of up- and downregulated genes in fish oil feeding compared with safflower oil feeding were 63 (6%) and 102 (10%), respectively.

Table 1. *Number of genes altered in high-fat diet*

		High fat	
	Carbohydrate	Safflower	Fish
Absolute call is present	1,669	1,925	1,928
Average difference is more than 150	975	1,039	992
Difference call is			
increase (vs. Carb.)		132	117
increase (vs. Saf.)			63
decrease (vs. Carb.)		14	48
decrease (vs. Saf.)			102

Total number of genes analyzed is 6,521. Absolute call, which was calculated by Affymetrix Gene Chip 3.1 software using several markers, is an indicator of the presence or absence of each gene transcript. Average difference value is a marker of abundance of each gene, obtained by comparing the intensity of hybridization to 20 sets of perfectly matched 25-mer oligonucleotide relative to 20 sets of mismatched oligonucleotide using this software. Difference call which defines genes that increased or decreased is derived from this software and is classified in 5 stages, that is, transcript has increased, decreased, marginally increased, marginally decreased, or has exhibited no change in expression level. Carb., carbohydrate-fed; safflower oil-fed.

Upregulated genes of fish oil feeding. Of 63 genes that upregulated in fish oil feeding compared with safflower oil feeding, 24 genes showed increase greater than twofold. In order of the fold increase, they are listed in Table 2 with their average differences (corresponding to expression level) and putative function. When they are classified into subgroups according to their functions, genes related to immune reaction, fat oxidation, and antioxidant emerge (Table 3). Enzymes related to fatty acid oxidation, such as fatty acid transport protein, Cyp 4a-10, long-chain fatty ACS, and carnitine palmitoyltransferase II, upregulated in fish oil-fed mice. Compared with carbohydrate-fed mice, Cyp 4a-10, long-chain fatty ACS also upregulated in safflower oil-fed mice, but their increase in safflower oil-fed mice was smaller than those in fish oil-fed mice. The different expression levels of these genes among three groups might be to the levels of PPARα activation. In fish oil-fed mice, testosterone 16-alpha-hydroxylase related to steroid degradation also upregulated by 4.3-fold. Upregulation of these genes is mostly mediated by PPARα activation (17). In other genes, alpha 2 type IV collagen, beta-tubulin, insulin-like growth factor-binding protein 2, and biglycan related to cell proliferation and formation of connective tissues also upregulated. However, their physiological roles of upregulation in fish oil feeding have not been elucidated.

Fish oil feeding upregulates immune-reacted genes in a long-term feeding period. Largest increases were observed in immune-reacted genes (Table 2). Components of immunoglobin such as immunoglobin kappa chain and immunoglobin heavy-chain constant region μ (b) markedly upregulated 37- and 24-fold, respectively. Because μ-type heavy chain is found in IgM, an increase of IgM is anticipated. Indeed, in rats, compared with safflower oil-fed group, fish oil feeding enhanced the serum IgM level (12). In relation to immune response, gelsolin involved in phagocytosis of neutrophils and macrophages (36) also upregulated by 10-fold. Leukocyte elastase inhibitor that inactivates enzymes related to immune reaction (34) also upregulated by ninefold. These immunological reactions observed in fish oil-fed mice were not due to hepatocyte injury, because there were no increases of serum glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) concentrations (data not shown).

Table 2. *Fish oil diet-induced increases in gene expression in liver*

	Accession Number	Fold Increase	Gene Description	Average Difference			Putative Function	Group
				Carbohydrate	Safflower	Fish		
1	V00802	37	Kappa-immunoglobulin (constant region)	27	14	1797 ↑	Inflammatory	1
2	X03690	24	Ig heavy-chain constant region mu(b) allele.	3	1	519 ↑	Inflammatory	1
3	J04953	10	Gelsolin	27	71	245 ↑	Phagocytosis	1
4	AA145127	8.9	Homologous to homosapiens leukocyte elastase inhibitor	52	67	600 ↑	Protease inhibitor	1
5	J04695	6.6	Alpha-2 type IV collagen	32	12	170 ↑	Matrix	
6	W29430	5.7	Hepatocyte growth factor activator inhibitor type 2	97	22	158 ↑	Protease inhibitor	
7	U15976	5.6	Fatty acid transport protein	32	45	253 ↑	Fatty acid transport	2
8	M23998	4.3	Testosterone 16-alpha-hydroxylase	109	121	440 ↑	Steroid hormone degradation	
9	U69135	4.1	Uncoupling protein 2	381	576	2415 ↑	Uncoupler	3
10	U48420	4.1	Theta class glutathione transferase type 2	68	206	840 ↑	Conjugation with glutathione	3
11	L06047	3.8	Glutathione transferase	68	59	222 ↑	Conjugation with glutathione	3
12	W29265	3.2	Glutathione transferase Ya subunit	221	169	616 ↑	Conjugation with glutathione	3
13	X69296	2.9	Cytochrome P-450, 4a10	412	1296 ↑	3813 ↑	Fatty acid omega oxidation	2
14	U15977	2.8	Long-chain fatty acyl CoA synthetase	306	1124 ↑	3941 ↑	Fatty acid beta oxidation	2
15	M32599	2.5	Glyceraldehyde-3-phosphate dehydrogenase	2873	783 ↓	2052 ↑	Glycolysis	
16	X70303	2.5	Proteosome subunit, alpha type 2	146	140	421 ↑	Protein turnover	
17	U15636	2.4	T cell specific GTPase, GTP-binding protein	139	123	334 ↑	Unknown	
18	AA028398	2.4	Beta-tubulin	218	106	255 ↑	Cytoskeleton, chaperone	
19	L05439	2.3	Insulin-like growth factor binding protein 2	690	923	1602 ↑	Modify IGF function	
20	X62940	2.3	Transforming growth factor-beta-stimulated clone-22	163	133	308 ↑	Transcription factor	
21	U01163	2.2	Carnitine palmitoyltransferase II	655	767	1653 ↑	Fatty acid beta oxidation	2
22	L20276	2.2	Biglycan	538	442	1090 ↑	Matrix	
23	L35528	2.1	Manganese superoxide dismutase	447	443	925 ↑	Antioxidant	3
24	Z38015	2.0	Myotonin protein kinase	368	289	574 ↑	Ca^{2+} homeostasis	

Group 1, immunoreaction; *group 2*, fat oxidation; *group 3*, antioxidant. In order of fold change levels in gene expression in liver from fish-oil fed mice relative to safflower-oil fed mice, genes increased more than 2-fold are presented. Fold change calculation was carried out as an indication of the relative change of each transcript represented on the probe array. The average difference value is a marker of abundance of each gene. Putative gene functions are based on literature review. Change (↑/↓) in fish oil fed mice indicates that difference call is significantly increased/decreased, compared with safflower oil-fed mice, while that in safflower oil-fed mice was compared with that in high-cabohydrate-fed mice.

Table 3. *Altered gene expression by fish oil feeding*

Increased	Decreased
Immune reaction ↑	Cholesterol and fatty acids synthesis ↓
Kappa-immunoglobulin (constant region)	Stearoyl-CoA desaturase
Ig heavy chain constant region mu(b) allele	Homologous to rat ATP citrate lyase
Gelsolin	Fatty acid synthase
Homologous to homo sapiens leukocyte elastase inhibitor	Squalene epoxidase
Fat oxidation ↑	Homologous to rat farnesyl pyrophosphate synthetase
Fatty acid transport protein	Low density lipoprotein receptor
Cytochrome p450,4a-10	Transcription ↓
Long chain fatty acyl CoA synthetase	Homologous to rat SREBP-1c
Carnitine palmitoyltransferase II	Silent mating type information regulation 2
Antioxidant ↑	ATF-4 gene for activating transcription factor 4
Uncoupling protein 2	Calcium binding protein P22
Theta class glutathione transferase type 2	Id2 protein
Glutathione transferase	ROS and PPARα activator production ↓
Glutathione transferase Ya subunit	Cytochrome p450 17-alpha hydroxylase/c17-20 lyase
Manganese superoxide dismutase	Hydroxysteroid sulfotransferase
	Phenol/aryl form sulfotransferase

Group of genes that altered in expression levels in fish oil feeding compared with safflower oil feeding is presented.

To examine whether upregulation of these genes by 6 mo, fish oil feeding was also observed in a short-term feeding period and was related to activation of PPARα, expression levels of immunoglobulin kappa chain, and gelsolin in fish oil-fed mice, and mice administered fenofibrate were examined by Northern blotting (Fig. 1). However, fish oil feeding did not upregulate these genes in 1 wk of feeding but markedly upregulated them in 6 mo of feeding. In addition, there was a large variation of immunoglobulin kappa chain expression levels of individual mice in fish oil-fed mice. Gene-chip analysis did not detect this individual variation, because it used a pooled sample. One week of fenofibrate administration did not upregulate these genes but rather downregulated gelsolin expression. Thus upregulation of these immune-related genes is not due to direct PPARα activation and may be related to chronic activation of neutrophils against oxidative stress.

Fish oil or fenofibrate administration upregulates antioxidant genes. In this gene-chip analysis, genes having antioxidant activities, such as uncoupling protein-2 (UCP-2), theta-class glutathione transferase type 2, glutathione transferase, glutathione transferase Ya subunit, and manganese superoxide dismutase (Mn-SOD) upregulated by two- to fourfold (Tables 2 and 3). UCP-2 and Mn-SOD, which are located in mitochondria, reduced superoxide formation (6, 26). A recent study of UCP-2 knockout mice revealed an important role of UCP-2 in macrophage-mediated immunity and ROS generation (1). Glutathione transferases in cytosol promoted conjugation of toxic electrophilic xenobiotics to glutathione (22). However, expression levels of other genes related to antioxidant systems, such as Cu-Zn SOD (M60798), cellular (AA123700) and plasma (U13705) glutathione peroxidase, and catalase (L25069), did not alter in fish oil-fed mice (data not shown).

To examine whether upregulation of these genes by 6-mo fish oil feeding was also observed in a 1-wk feeding period and was related to activation of PPARα, expression levels of these genes in fish oil-fed mice and fenofibrate-administered mice were examined by Northern blotting. Because it has been previously reported that fish oil and fenofibrate upregulate UCP-2

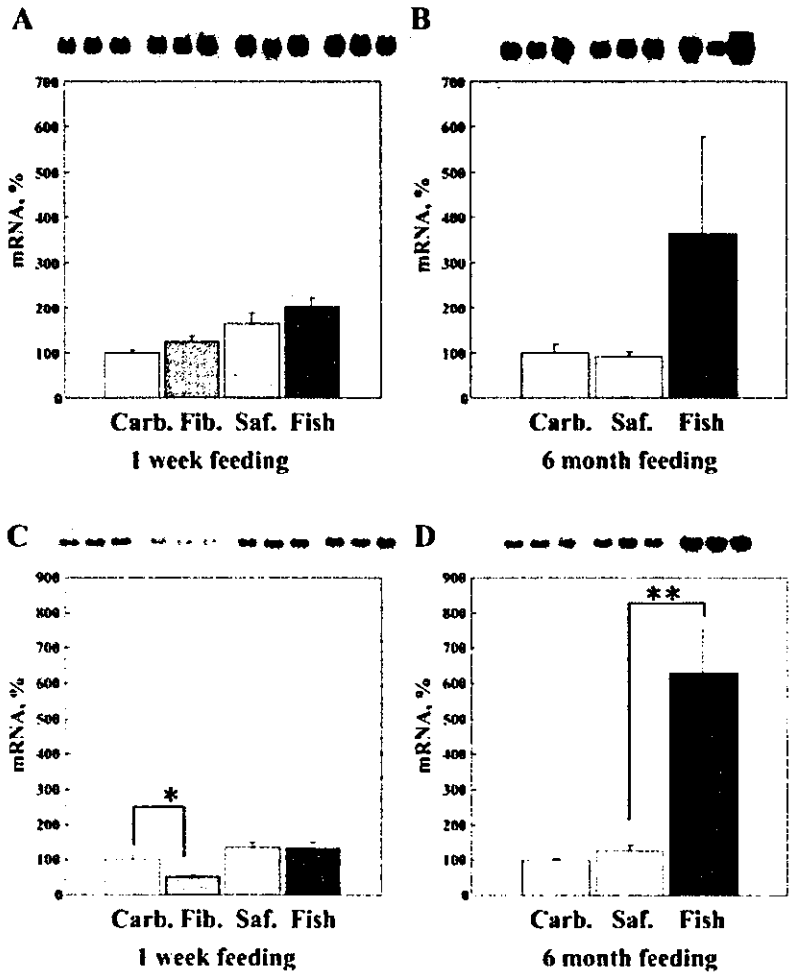

Fig. 1. Northern blotting for kappa-immunoglobulin (*A*, *B*) and gelsolin (*C*, *D*) from livers from carbohydrate (Carb.)-, fenofibrate-administered carbohydrate (Fib.)-, and safflower oil (Saf.)- and fish oil-fed mice (Fish) for 1 wk (*A*, *C*) and 6 mo (*B*, *D*). Total RNA was isolated from livers of carbohydrate diet, fenofibrate-administered carbohydrate diet (for 1 wk only), and safflower oil diet- and fish oil diet-fed mice at 1 wk feeding (*A*, *C*) and 6 mo feeding (*B*, *D*). Fifteen-microgram aliquots of total RNA were subjected to electrophoresis and transferred to Nylon membranes. The membranes were hybridized with ^{32}P-labeled probe for kappa-immunoglobulin (*A*, *B*) and gelsolin (*C*, *D*). In autoradiogram, each line represents a sample from an individual mouse. The radioactivity in each band was quantified using an image analyzer. The data for each band are shown in values relative to the mRNA level of carbohydrate diet group mice. In *A* and *B*, a typical autoradiogram of kappa-immunoglobulin (24-h exposure) and its relative levels are shown. In *C* and *D*, a typical autoradiogram of gelsolin (24-h exposure) and its relative levels are shown. Each value represents a mean ± SE of 5 mice. Statistical differences are shown: $^{*}P < 0.05$ and $^{**}P < 0.01$.

mRNA (38), glutathione transferase Ya subunit and Mn-SOD (4k and 1k) mRNAs were presented (Fig. 2). In agreement with gene-chip analysis, compared with safflower oil feeding, fish oil feeding also increased these enzyme expressions by 3.8-fold ($P < 0.001$), 1.3-fold (4k, $P < 0.001$), and 1.9-fold (1k, $P < 0.001$), respectively. Administration of 0.5% (wt/wt) fenofibrate for 1 wk increased glutathione transferase Ya subunit and Mn-SOD mRNAs expression by 2.2-fold ($P < 0.01$), 1.4-fold (4k, $P < 0.001$) and 2.0-fold (1k, $P < 0.001$), respectively. In addition, 1-wk administration of fenofibrate also upregulated gene expression of glutathione transferase theta-class type 2 (data not shown). Thus upregulation of glutathione transferases and Mn-SOD by fish oil feeding is also mediated by PPARα activation.

Downregulated genes in fish oil feeding. Of 102 genes that downregulated in fish oil feeding compared with safflower oil feeding, 25 genes showed a decrease less than twofold. In order of fold decrease, they are listed in Table 4 with their average differences and putative function. Of 25 genes, 6 genes were related to cholesterol and fatty acid synthesis. They were stearoyl-CoA desaturase, ATP citrate-lyase, fatty acid synthase, squalene epoxidase, farnesyl pyrophosphate synthetase, and low-density lipoprotein receptor. In agreement with our previous findings (15), parallel with decreases of these enzymes, a marked reduction of SREBP-1c may be responsible for downregulation of cholesterol and fatty acid synthesis.

In addition, fish oil feeding downregulates several transcription factors, silent mating-type information regulation 2 (Sir 2), ATF-4, P22, and Id2 against safflower oil feeding. However, Sir 2, ATF-4, and P22 in fish oil diet did not differ against carbohydrate diet; i.e., these transcription factors upregulated in safflower oil diet relative to carbohydrate diet. Sir2 encodes a protein that promotes compact chromatin structure, thereby preventing or silencing gene transcription at selected levels. Recently, calorie restriction in yeast failed to extend lifespan in strains mutant for Sir2 (20). Thus the increased longevity-induced calorie restriction requires Sir2 protein. An increase of Sir2 in safflower oil feeding might increase lifespan of hepatocytes. Activation transcription factor 4 interacts with many transcription factors and coactivators such as TATA-binding protein, TFIIB, RAP 30 subunit of TFIIF, and CREB-binding protein (19). Id2 (inhibitor of DNA binding) has recently been shown to bind SREBP-1c and inhibit transcription of fatty acid synthase (24). Thus reduction of Id2 might be an adaptive response against a marked decrease of SREBP-1c mRNA. Further studies are necessary to clarify physiological roles of these transcription factors in high-fat diet.

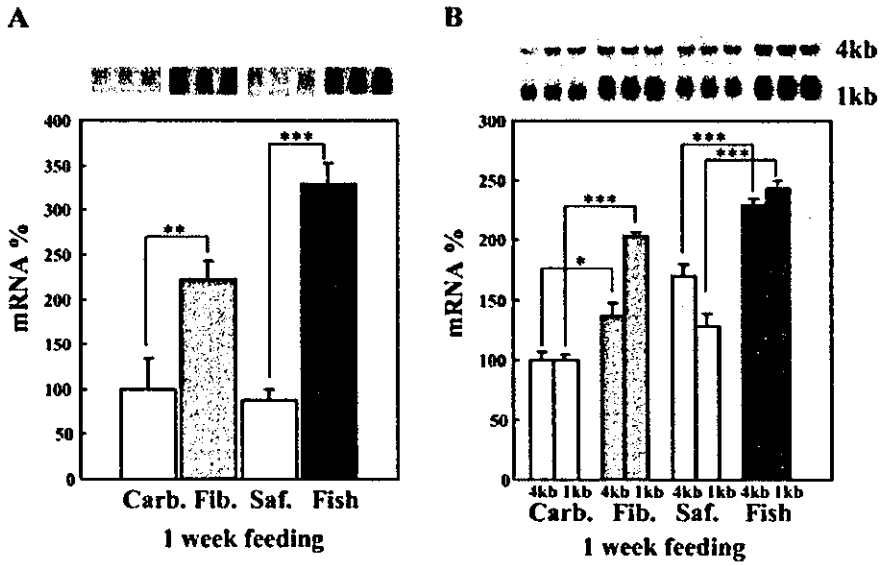

Fig. 2. Northern blotting for glutathione transferase Ya subunit (*A*) and Mn-superoxide dismutase (*B*) from livers from carbohydrate-, fenofibrate-administered carbohydrate-, and safflower oil- and fish oil-fed mice for 1 wk. Total RNA was isolated from livers of carbohydrate diet, fenofibrate-administered carbohydrate diet, and safflower oil diet- and fish oil diet-fed mice at 1 wk feeding. Fifteen-microgram aliquots of total RNA were subjected to electrophoresis and transferred to Nylon membranes. The membranes were hybridized with ^{32}P-labeled probe for glutathione transferase Ya subunit and Mn-superoxide dismutase. In autoradiogram, each line represents a sample from an individual mouse. The radioactivity in each band was quantified using an image analyzer. The data for each band are shown in values relative to the mRNA level of carbohydrate diet group mice. In *A*, a typical autoradiogram glutathione transferase Ya subunit (24-h exposure) and its relative levels are shown. In *B*, a typical autoradiogram of Mn-superoxide dismutase (24-h exposure) and their relative levels (4 and 1 kb) are shown. Each value represents a mean ± SE of 5 mice. Statistical differences are shown: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Table 4. *Fish oil diet-induced decreases in gene expression in liver*

	Accession Number	Fold Change	Gene Description	Average Difference			Putative Function	Group
				Carbohydrate	Safflower	Fish		
1	M21285	−83	Stearoyl-CoA desaturase gene	13098	7644	81 ↓	PUFA synthesis	1
2	L27121	−34	Hydroxysteroid sulfotransferase	952	1330 ↑	3 ↓	Phase II xenobiotic metabolising enzymes	2
3	M64863	−29	Cytochrome P-450 17-alpha hydroxylase/C17-20 lyase	1143	952	−77 ↓	Extra glandular steroid genesis	2
4	W89667	−15	Homologous to rat sterol regulatory element binding protein −1c	266	181	14 ↓	Transcription factor	3
5	L41631	−9.2	Glucokinase	375	338	4 ↓	Glucose uptake	
6	W17745	−7.2	Homologous to rat ATP Citrate-lyase	2815	1647 ↓	229 ↓	Fatty acid synthesis	1
7	AA137659	−6.5	Cytochrome P-450 IIC40	1505	1652	252 ↓	Inflammatory	
8	AA139907	−5.8	Spot14	1182	269 ↓	46 ↓	Unknown	
9	X13135	−5.7	Fatty acid synthase	2781	1505 ↓	275 ↓	Fatty acid synthesis	1
10	M64250	−5.4	Apolipoprotein A-IV	331	275	71 ↓	HDL cholesterol metabolism	
11	W48402	−4.3	Silent mating type information regulation 2	78	212 ↑	50 ↓	Silencing gene transcription in yeast	3
12	D42048	−4.1	Squalene epoxidase	364	539 ↑	114 ↓	Cholesterol synthesis	1
13	X05475	−3.8	Complement component C9	919	753	229 ↓	Cell lysis	
14	AA036251	−3	Homologous to rat farnesyl pyrophosphate synthetase	385	403	156 ↓	Cholesterol synthesis	1
15	W81960	−2.5	Phenol/aryl form sulfotransferase	764	722	249 ↓	Phase II xenobiotic metabolising enzymes	2
16	M33212	−2.5	Nucleolar protein N038	113	304 ↑	121 ↓	Unknown	
17	M58588	−2.5	Plasma kallikrein	160	289	117 ↓	Blood coagulation	
18	U51805	−2.4	Arginase	2206	2641	1124 ↓	Urea production	
19	M73329	−2.3	Phospholipase C-alpha	535	1057 ↑	424 ↓	Chaperone	
20	M94087	−2.3	ATF-4 gene for activating transcription factor 4	568	800 ↑	351 ↓	Transcription factor	3
21	M19960	−2.3	cAMP-dependent protein kinase catalytic subunit	192	341 ↑	146 ↓	Protein kinase A	
22	M16359	−2.2	Major urinary protein III	3491	4110	2144 ↓	Unknown	
23	X64414	−2.2	Low-density lipoprotein receptor	436	602	271 ↓	Lipid intake	1
24	W47892	−2.2	Calcium binding protein P22	226	446 ↑	182 ↓	Protein phosphatase	3
25	M69293	−2.1	Id2 protein	608	703	340 ↓	Transcription factor, inhibit bHLH transcription factors	3

Group 1, cholesterol and fatty acid synthesis; *group 2,* reactive oxygen species (ROS) and peroxisomal proliferator-activated receptor α (PPARα) activator production; *group 3,* transcription. HDL, high-density lipoprotein; PUFA, polyunsaturated fatty acids. In order of fold change levels in gene expression in liver from fish oil-fed mice relative to safflower oil-fed mice, genes change less than 2-fold are presented. Fold change calculation was carried out as an indication of the relative change of each transcript represented on the probe array. The average difference value is a marker of abundance of each gene. Putative gene functions are based on literature review. Some of genes are classified by its functions as "↑/↓" in fish oil-fed mice indicates that difference call is significantly increased/decreased, compared with safflower oil-fed mice, while that in safflower oil-fed mice was compared with that in high-carbohydrate-fed mice.

Fish oil or fenofibrate administration downregulates genes related to induction of endogenous PPARα activator. Fish oil feeding downregulated the expression of cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase and sulfotransferases (Tables 3 and 4). Cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase catalyzes formation of dehydroepiandrosterone (DHEA) from pregnenolone (25). Because the sulfated form of DHEA by hydroxysteroid sulfotransferase is a peroxisomal proliferator (39), marked reduction of both 17-alpha hydroxylase/C17–20 lyase and hydroxysteroid sulfotransferase may be an adaptive response to decrease production of endogenous PPARα activators. In addition, hydroxysteroid and phenol/aryl form sulfotransferases produce ROS (10). Sulfation by sulfotransferase is a common final step in the biotransformation of xenobiotics and is traditionally associated with inactivation. However, the sulfate group is electron withdrawing and may be cleaved off heterolytically in some molecules, leading to electrophilic cation (10).

The mechanism(s) of downregulation of 17-alpha hydroxylase/C17–20 lyase and sulfotransferases were different. Fenofibrate administration did not downregulate 17-alpha hydroxylase/C17–20 lyase mRNA but downregulated hydroxysteroid and phenol sulfotransferases mRNAs by 88% ($P < 0.05$) and 83% ($P < 0.001$), respectively, whereas fish oil feeding for 1 wk downregulated all these enzymes (Fig. 3). Thus downregulation of hydroxysteroid and phenol sulfotransferases by fish oil feeding is also mediated through PPARα activation, but 17-alpha hydroxylase/C17–20 lyase is not. Because 17-alpha hydroxylase/C17–20 lyase is an enzyme for steroid catabolism, it may be downregulated by a decrease of mature form of SREBP-1c (15).

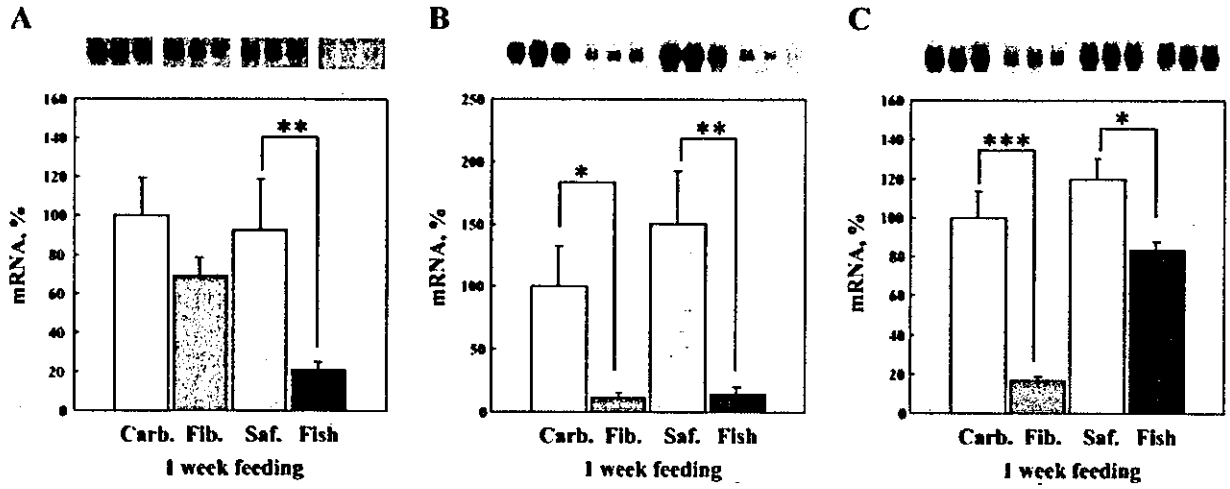

Fig. 3. Northern blotting for cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase (*A*), hydroxysteroid sulfotransferase (*B*), and phenol/aryl-form sulfotransferase (*C*) from livers from carbohydrate-, fenofibrate-administered carbohydrate-, and safflower oil- and fish oil diet-fed mice for 1 wk. Total RNA was isolated from livers of carbohydrate diet-, fenofibrate-administered carbohydrate diet-, and safflower oil- and fish oil diet-fed mice at 1 wk feeding. Fifteen-microgram aliquots of total RNA were subjected to electrophoresis and transferred to Nylon membranes. The membranes were hybridized with ^{32}P-labeled probe for cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase, hydroxysteroid and phenol/aryl form sulfotransferases mRNAs. In autoradiogram, each line represents a sample from an individual mouse. The radioactivity in each band was quantified using an image analyzer. The data for each band are shown in values relative to the mRNA level of carbohydrate diet group mice. In *A*, typical autoradiogram of cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase (24-h exposure) and its relative levels are shown. In *B*, typical autoradiogram of hydroxysteroid sulfotransferase mRNAs (48-h exposure) and its relative levels are shown. In *C*, a typical autoradiogram of phenol/aryl-form sulfotransferase mRNAs (48-h exposure) and its relative levels are shown. Each value represents the mean ± SE of 5 mice. Statistical differences are shown: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

DISCUSSION

By using gene-chip analysis, we have studied genes that were markedly altered in expression levels by fish oil feeding. Although we have selected some genes in limited number of genes, we could show not only that the well-known genes related to fatty acid oxidation and triglyceride synthesis, but also new classes of genes related to PPARα activation and ROS production are markedly altered by fish oil feeding.

Fatty acids, particularly eicosanoids and their metabolites (42), constitute one class of endogenous PPARα activators, whereas steroid DHEA and its metabolites constitute another class of naturally occurring PPARα activators. When DHEA is administrated to rodents, this androgen precursor acts as a strong peroxisome proliferator (7, 40) and inducer of liver peroxisomal enzyme expression and Cyp 4A gene transcription (31). However, DHEA is effective in inducing each gene of these mRNAs in rat liver in vivo (31), but this steroid has no effect in cultured cells (32). This suggests that DHEA per se is not an active inducer but that this metabolite is more active inducer. In rat primary hepatocytes, DHEA-3β sulfate (DHEA-S), a DHEA metabolite produced by steroid sulfation (14), increases in peroxisomal enzyme and Cyp 4A expression levels at physiologically relevant concentrations of DHEA (10 μM) (32). In addition, because DHEA-S is unable to induce a liver peroxisome proliferative response in vivo when administrated to PPARα gene knockout mice (30), it is evident that peroxisome proliferative response by DHEA-S is mediated by PPARα. Our finding that fish oil feeding resulted in marked reductions of two important enzymes for DHEA-S formation, namely 17-alpha hydroxylase/C17–20 lyase that catalyzes the conversion of pregnenolone to DHEA and hydroxysteroid sulfotransferase that catalyzes the conversion of DHEA to DHEA-S, indicates an operation of a negative-feedback system to reduce endogenous ligand formation for PPARα activation against increased activation of PPARα caused by fish oil feeding (Fig. 4). Thus fish oil-mediated PPARα activation may inhibit steroid-mediated PPARα activation to prevent some PPARα-mediated deteriorative effects such as ROS production and cancer formation (41). However, to prove this hypothesis, it is necessary to identify endogenous ligands for PPARα activation and to show that fish oil feeding results in a reduction of PPARα ligands derived from the steroid pathway.

Sulfotransferase is not only related to PPARα activation but also to ROS production. Sulfotransferases are phase II drug-metabolizing enzymes that sulfoconjugate a variety of endogenous and exogenous compounds such as biogenic amines, steroid hormones, bile acids, drug, and carcinogens (11, 16). However, the sulfate group is electron withdrawing and may be cleaved off heterolytically in some molecules, leading to an electrophilic cation (10). Fish oil feeding and fenofibrate administration downregulate hydroxysteroid

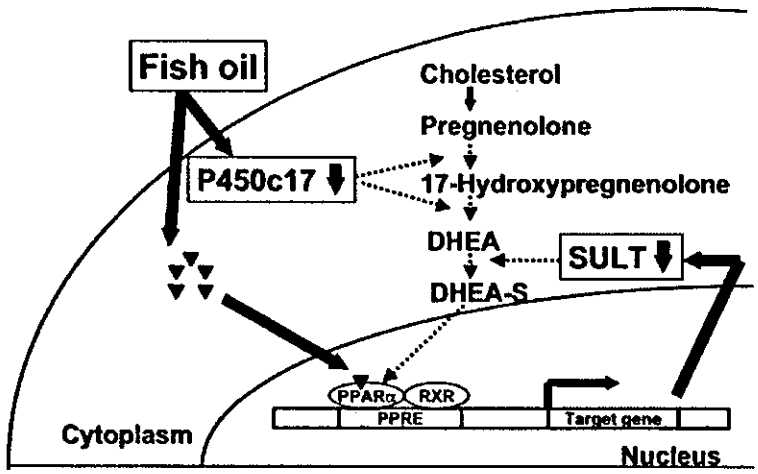

Fig. 4. A proposed model for the negative feedback of peroxisomal proliferator-activated receptor α (PPARα) activation. Fatty acids, in particular, eicosanoids and their metabolites, constitute 1 class of endogenous PPARα activators, whereas adrenal steroid dehydroepiandrosterone (DHEA) and its metabolites constitute another class of naturally occurring PPARα activators. Cytochrome *P*-450 17-alpha hydroxylase/C17–20 lyase (P450c17) catalyzes formation of DHEA. Because sulfated form of DHEA (DHEA-S) by hydroxysteroid sulfotransferase (SULT) is a peroxisomal proliferator (30), marked reduction of both P450c17 and SULT by fish oil feeding may be an adaptive response against fish oil-induced PPARα activation. Decreased mRNA of SULT is PPARα dependent, whereas that of P450c17 is not.

and phenol/aryl form sulfotransferase. Both types of sulfotransferases are related to ROS production. Thus downregulated hydroxysteroid and phenol/aryl form sulfotransferase by fish oil feeding may contribute to a decrease of ROS production.

In this line of evidence, fish oil feeding and fibrate administration upregulated antioxidant genes such as UCP-2, glutathione transferase, and Mn-SOD. Glutathione S-transferases (GST) are a multigene family; four classes of protein, α, μ, π, and θ, have been demonstrated (2). In mouse, α-class GST consists of two subunits Ya and Yc. Glutathione transferase (L06047) in Table 2 corresponds to α4-type, which abundantly expresses in lung tissues (43). Ya subunit gene expression is induced by planar aromatic compounds and electrophiles (8), and antioxidant response element is found in the 5′-flanking region of this gene (28). Fish oil feeding upregulated Ya, α4-, and θ-types of GST to quench ROS in cytosol, whereas it upregulated UCP-2 and Mn-SOD to quench ROS in mitochondria (Fig. 5).

Because fish oil-mediated alterations of gene expression related to ROS production were also observed in fenofibrate-administered mice, it is conceivable that alterations of these genes are mediated by PPARα activation. However, altered gene expressions observed in fenofibrate administration are due to the direct effects mediated by peroxisome proliferator responsive elements or the indirect effects of other *cis*-elements by PPARα-activated gene products or PPARα-unrelated effects. Most of the fenofibrate effects are considered to be mediated directly and indirectly by PPARα (17, 18), but it is not ruled out that in case of some genes, similar alterations of gene expression between fenofibrate administration and fish oil feeding were merely coincident and were not related to PPARα activation.

In this study, to clarify the effects of fish oil feeding, we used a very high-fat diet (60% of total energy intake). We have observed that there was a linear, dose-dependent effect of fish oil on upregulation of

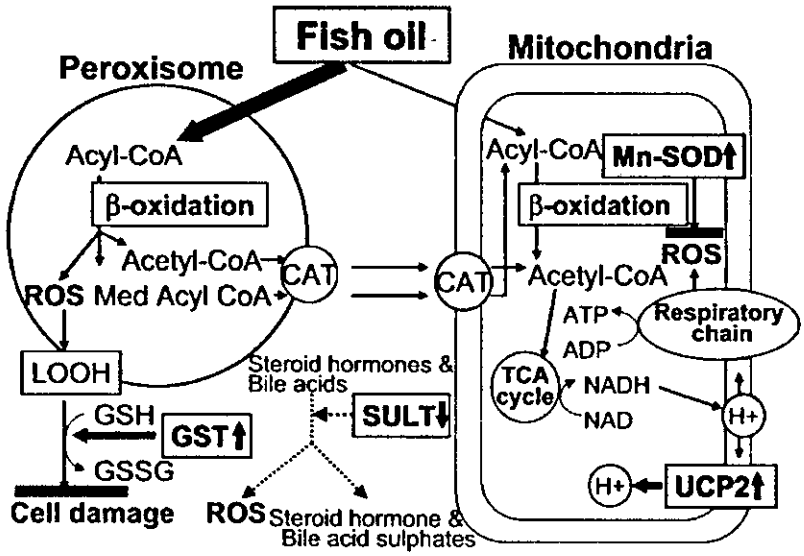

Fig. 5. A proposed model of antioxidant system in fish oil diet. Long-chain unsaturated fatty acids from fish oils are initially metabolized to acetyl-CoA and medium-chain acyl-CoA (Med acyl-CoA) in peroxisome by β-oxidation. Reactive oxygen species (ROS) and peroxidized lipid (LOOH) generated in this process are quenched glutathione *S*-transferase (GST) in cytosol. Acetyl-CoA and Med acyl-CoA are transferred to mitochondria by carnitine acetyltransferase (CAT). Med acyl-CoA is metabolized into acetyl-CoA, and acetyl-CoA is used in the tricarboxylic acid cycle. ROS produced in respiratory chain is reduced by uncoupling protein 2 (UCP-2) through a reduction proton gradient and also quenched by Mn-superoxide dismutase (Mn-SOD). In addition, sulfation of bile acids and steroid hormones by SULT generates ROS. A reduction of SULT may also contribute to a reduction of ROS formation.

PPARα-activated genes such as ACS, LPL, and UCP-2 mRNAs; 10% of fish oil showed a significant increase of ACS, LPL, and UCP-2 mRNAs (data not shown). Thus we expected that alterations of gene expression observed in 60% oil diet would also be observed in the range of daily intake of fish.

Another finding of gene-chip analysis is that immunological reaction was observed in fish oil feeding, but this effect was not observed in PPARα activator. Because lipid peroxidation products, which are specific to fish oil feeding, are a potent chemostatic factor for neutrophils (5), it is possible that fish oil feeding increases lipid peroxidation products and induces neutrophil migration and activation in liver tissues. Thus increased immunological reaction as well as induction of antioxidant genes might be an adaptive reaction against increased oxidative stress.

In summary, gene-chip analysis revealed that activators of PPARα, such as fish oil and fenofibrate, altered gene expression profiles to defend against excess PPARα activation and ROS production.

We are grateful to NOF for the supply of fish oil and to Dr. Nakagawa at Kitasato University for the supply of rat GST Ya subunit cDNA and to Dr. Ookawara at Hyogo Medical University for the supply of rat Mn-SOD cDNA.

This work was supported in part by Special Coordination Funds for Promoting Science and Technology from the Japanese Science and Technology Agency (Tokyo) and by research grants from the Japanese Ministry of Health, Labor, and Welfare (Tokyo) and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (Tokyo).

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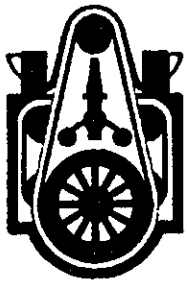

American Journal of Pathology, Vol. 160, No. 3, March 2002

Frequent Co-Localization of Cox-2 and Laminin-5 γ2 Chain at the Invasive Front of Early-Stage Lung Adenocarcinomas

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Laminin-5 is an extracellular matrix protein that plays a key role in cell migration and tumor invasion. Cox-2 is an induced isoform of cyclooxygenases that plays an important role in carcinogenesis, suppression of apoptosis, angiogenesis, and metastasis of colon cancer. We report frequent co-expression of cox-2 and laminin-5 at the invasive front of early-stage lung adenocarcinomas. We investigated the expression of cox-2 and laminin-5 immunohistochemically in 102 cases of small-sized lung adenocarcinoma (maximum dimension, 2 cm or less). Cox-2 and laminin-5 were expressed in 97 (95.1%) and 82 (80.4%) cases, respectively. Both were preferentially localized in cancer cells at the cancer-stroma interface, although cox-2 tended to show a diffuse staining pattern in some cases. A comparison of their staining patterns revealed a striking similarity in their distribution in 24 cases, and a partial overlap between their localization in another 20 cases. Moreover, an overall correlation was found between the expression levels of cox-2 and laminin-5 ($P = 0.018$). To gain insight into the mechanisms that regulate the expression of these proteins, we additionally studied their expression in 58 cases of stage I lung adenocarcinoma, in which p53 status was determined by immunohistochemistry, polymerase chain reaction-single strand conformation polymorphism analysis, and direct sequencing. The results showed that tumors with mutant p53 tended to express more cox-2 than those with wild-type p53 ($P = 0.080$). Also, tumors that overexpressed p53 had higher levels of cox-2 and laminin-5 than those without p53 overexpression ($P = 0.032$ and 0.047, respectively). Further immunohistochemical analysis showed that tumors that overexpressed both epidermal growth factor receptor (EGFR) and erbB-2 had higher levels of cox-2 and laminin-5 than those without concomitant overexpression of these proteins ($P = 0.014$ and $P = 0.018$, respectively). To see whether EGFR signaling is involved in cox-2 and laminin-5 expression, we further conducted *in vitro* analyses using six lung adenocarcinoma cell lines (A549, HLC-1, ABC-1, LC-2/ad, VMRC-LCD, and L27). Western blot analyses showed that cox-2 mRNA levels, and to a lesser extent laminin-5 γ2 mRNA levels, correlated with the expression levels of erbB-2 and the phosphorylated form of MAPK/ERK-1/2 protein. The addition of transforming growth factor-α increased both cox-2 and laminin-5 γ2 mRNA levels in A549, ABC-1, and L27 with different kinetics; the induction of cox-2 occurred earlier than that of laminin-5 γ2. Finally, the migration of ABC-1 cells was inhibited by MAP kinase kinase inhibitor PD98059 and a selective cox-2 inhibitor NS-398. In contrast, the migration of A549 cells was inhibited by PD98059, but much less effectively by NS-398. These results suggest that co-stimulatory mechanisms may exist that increase the expression of cox-2 and laminin-5 at the invasive front of lung adenocarcinomas and that EGFR signaling could be one of the mechanisms. Further investigations are warranted concerning the role of cox-2 and laminin-5 in cancer cell invasion and the significance of p53 and EGFR signaling in the regulation of cox-2 and laminin-5 expression. ***(Am J Pathol 2002, 160:1129–1141)***

Cyclooxygenases are rate-limiting enzymes that catalyze the conversion of arachidonic acid to prostaglandins.[1–4] Cyclooxygenases exist in two isoforms: a constitutively expressed isoform, cox-1, and an induced isoform, cox-2. The expression of cox-2 is induced by cytokines

Supported by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan and Grants-in-Aid from the Ministry for Health and Welfare and from the Ministry of Education, Science, Sports, and Culture of Japan.

Accepted for publication December 20, 2001.

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and growth factors. Numerous clinical and experimental studies have suggested that cox-2 plays an important role in carcinogenesis, suppression of apoptosis, angiogenesis, and metastasis of colon cancer.[1–4] Overexpression of cox-2 is frequently observed in colon adenoma and carcinoma.[5–7] Inhibition of cox-2 reduced colon adenoma formation in experimental animals[8,9] and patients with familial adenomatous polyposis.[10] Specific inhibitors of cox-2 reduced tumor growth *in vivo*[11] and induced apoptosis in tumor cells both *in vitro* and *in vivo*.[12–14] Moreover, introduction of cox-2 cDNA increased the production of metalloproteinases and a variety of angiogenic factors[15,16] and enhanced the metastatic potential of colon cancer cells.[15]

Overexpression of cox-2 has been documented in various other cancers,[17–22] including lung cancer.[23–25] Among the four histological types of lung cancer, cox-2 is most frequently expressed in adenocarcinoma.[23,24] In patients with stage I lung adenocarcinoma, overexpression of cox-2 is associated with poor prognosis.[25] Cox-2 inhibitors inhibited proliferation and induced apoptosis in various lung cancer cell lines.[26] Although these studies suggested the involvement of cox-2 in invasion and metastasis of lung adenocarcinoma, the underlying mechanism for the overexpression of cox-2 in these tumors is currently unclear. Results of culture studies suggest that cox-2 is induced by epidermal growth factor receptor (EGFR) signaling,[27,28] interleukin (IL)-1,[29,30] tumor necrosis factor-α,[31] and the activated H-ras oncogene.[32] A recent study by Subbaramaiah and colleagues[33] suggests a potential role of p53 in suppressing the expression of cox-2. However, it is currently unclear which of these factors are actually involved in the up-regulation of cox-2 in primary cancers.

We have recently reported that the laminin-5 γ2 chain is frequently overexpressed at the invasive front of small-sized lung adenocarcinomas (maximum dimension, 2 cm or less), and that overexpression of the laminin-5 γ2 chain is associated with poor patient prognosis.[34] Laminin-5 consists of three subunits, the α3, β3, and γ2 chains, the latter two being unique to this isoform. Laminin-5 is an extracellular matrix protein that plays a key role in cell migration and tumor cell invasion.[35–38] Several previous studies have shown that laminin-5 is frequently expressed at the invasive front of several cancers, including colorectal,[39–41] gastric,[42] pancreatic,[43] breast adenocarcinomas,[39] uterine cervical[39,40,44] and oral[45–47] squamous cell carcinomas, and malignant melanoma.[39,40] However, the regulatory mechanism for the overexpression of laminin-5 in cancer is currently unclear.

In this study, we report that cox-2 and laminin-5 are frequently co-localized at the invasive front of early-stage lung adenocarcinomas. We also present data showing that overexpression of cox-2 and laminin-5 is associated with p53 abnormalities and concomitant overexpression of EGFR and erbB-2. Finally, the results of our *in vitro* experiments also support the hypothesis that EGFR signaling is involved in the aberrant expression of cox-2 and laminin-5 in lung adenocarcinomas.

Materials and Methods

Patients and Tumors

We analyzed two groups of early-stage lung adenocarcinomas. First, we investigated the expression of cox-2 and laminin-5 immunohistochemically in 102 cases of small-sized lung adenocarcinoma (maximum dimension, 2 cm or less) that were resected at the National Cancer Center Hospital between 1984 and 1991. We recently reported the expression of laminin-5 and its prognostic significance in these small-sized adenocarcinomas.[34] The clinicopathological features of these patients and tumors are detailed in that report. Second, we examined the expression of cox-2 and laminin-5 immunohistochemically in 58 cases of stage I lung adenocarcinoma resected at the same hospital between 1985 and 1994, and analyzed its relationships with p53 abnormalities and the expression of EGFR and erbB-2. The p53 status of these adenocarcinomas was extensively characterized throughout the coding regions (exons 2 to 11), along with that of other non-small cell carcinomas (squamous cell, adenosquamous, and large-cell carcinomas), by polymerase chain reaction-single strand conformation polymorphism analysis, and direct sequencing.[48] Most of these 58 tumors were resected after 1990 and exceeded 2 cm in maximum dimension; therefore, there was no overlap between the two groups of tumors. Additionally, the distributions of laminin-5 α3, β3, and γ2 chains were studied in 20 cases of lung adenocarcinoma resected at Tokyo University Hospital between 2000 and 2001. Histological typing of the tumors was based on the new World Health Organization classification.[49] Disease stage was determined according to the TNM classification of the International Union Against Cancer.[50] The tumor tissues were routinely fixed in 10% formalin and embedded in paraffin.

Immunohistochemistry

Tissue blocks were cut into 4-μm-thick sections and deparaffinized through graded xylene and ethanol series. The sections were then washed in phosphate-buffered saline (pH 7.4), and treated with 0.3% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, sections were heated for 10 minutes at 120°C by autoclave treatment (for cox-2, laminin-5, p53, and erbB-2) or digested with 0.1% protease type XXVII (Sigma, St. Louis, MO, USA) for 20 minutes at room temperature (for EGFR). After incubation for 10 minutes with 10% normal swine serum to block nonspecific binding of the antibodies, the sections were incubated with rabbit polyclonal anti-cox-2 antibody (at a concentration of 2 μg/ml; IBL, Gunma, Japan), mouse monoclonal anti-laminin-5 γ2 chain antibody[46] (at a concentration of 1 μg/ml), mouse monoclonal anti-p53 antibody (at a dilution of $\times$1/100, clone DO-7; Novocastra, Newcastle-upon-Tyne, UK), mouse monoclonal anti-EGFR (at a dilution of $\times$1/10, clone 31G7; Zymed), or rabbit polyclonal anti-c-erbB-2 (at a concentration of 0.25 μg/ml; Nichirei, Tokyo, Japan). After overnight incubation with the primary antibody at 4°C,

Table 1. Sources of Antibodies to Laminin-5 Subunits Used in this Study

Laminin subunit	Source	Species	Dilutions
Laminin α3	Chemicon	Mouse monoclonal (clone P3H9-2)	×1/200
Laminin α3	Chemicon	Mouse monoclonal (clone P3E4)	×1/200
Laminin β3	Transduction Laboratories	Mouse monoclonal (clone 17)	×1/4,000
Laminin β3	Santa Cruz	Goat polyclonal	×1/50
Laminin γ2	Chemicon	Mouse monoclonal (clone D4B5)	×1/50
Laminin γ2	Pathology Division, NCCRI	Mouse monoclonal (clone 1-97)[53]	1 μg/ml

NCCRI, National Cancer Center Research Institute.

the sections were reacted with biotinylated secondary antibody for 45 minutes. Subsequently, the sections were allowed to react for 30 minutes with avidin-biotin-peroxidase complex (ABC) by using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and subjected to the peroxidase reaction with 0.02% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen in Tris-HCl buffer (pH 7.6) containing 0.007% hydrogen peroxide. No significant staining was observed in the negative controls, which were prepared by using the same class of mouse immunoglobulin at the same concentration.

We additionally used five commercially available antibodies against each subunit of laminin-5, ie, α3, β3, and γ2 chains. Immunohistochemistry was performed after antigen retrieval by autoclave treatment as described above for other antibodies. The sources and dilutions used for these five antibodies are summarized in Table 1.

Immunohistochemical Evaluation

Tumor cells were often heterogeneous with respect to cox-2 and laminin-5 expression, even within the same tumor (see below). Therefore, the expression of cox-2 and laminin-5 was graded by using the following method.[34] First, sections were scanned at low magnification to identify the area showing the highest level of expression. Then, that area was viewed with a ×10 objective, and the expression levels were graded on a scale of 0 to 2+ as follows: 0, either no positive cancer cells present or only a few scattered positive cancer cells; 1+, cluster(s) of positive cancer cells present, but accounting for less than 30% of the tumor cells within the visual field; 2+, cluster(s) of positive cancer cells that accounted for more than 30% of the tumor cells within the visual field. The grading for cox-2 was independently performed without previous knowledge of the grading for laminin-5, and *vice versa*. Overexpression of EGFR and erbB-2 was judged positive when most cancer cells (>50%) showed clear membranous staining. Only membranous staining was evaluated; cytoplasmic staining was not taken into account in evaluating EGFR or erbB-2 staining. Overexpression of p53 was evaluated as positive when more than 20% of the tumor cells showed nuclear staining.

Cell Lines and Culture

Lung adenocarcinoma cell lines A549, VMRC-LCD, and ABC-1 were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan), and HLC-1 and LC-2/ad from the RIKEN Cell Bank (Tsukuba, Japan). Lung adenocarcinoma cell line L27 was established in our laboratory. All cell lines were maintained in culture with Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, glutamine, and antibiotics, in a humidified atmosphere with 5% CO_2 and 95% air. To investigate the effect of transforming growth factor (TGF)-α on the expression of cox-2 and laminin-5 γ2 mRNA, preconfluent culture was washed and the culture media were replaced with serum-free Dulbecco's modified Eagle medium containing 0.2% bovine serum albumin. After culture of the cells in the serum-free media for 18 hours, recombinant human TGF-α was added to a final concentration of 50 ng/ml, and cells were lysed for RNA extraction at 1, 3, 8, and 24 hours after addition of TGF-α.

RNA Extraction

Total RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany). All samples were treated with RNase-free DNase (Qiagen) during the isolation, following the manufacturer's protocol. The purity and concentration of RNA were determined by spectrometry at 260 nm.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR was performed by using the SYBR green system as described previously.[51] Briefly, 2 μg of total RNA was reverse transcribed by using random hexanucleotide primers and SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). To prevent the reamplification of carryover PCR products, cDNAs were first treated with AmpErase uracil-*N*-glycosylase (Perkin-Elmer Biosystems, Valencia, CA, USA). The PCRs were performed by using the SYBR Green Core Reagents kit (Perkin-Elmer). The PCR amplification was performed by using a 96-well optical tray and caps in a final reaction volume of 50 μl. We used the PCR cycle parameters as recommended by the manufacturer's protocol. Real-time detection of the amplified cDNA was performed by using a Gene Amp 7700 Sequence Detection System (Perkin-Elmer).

The following oligonucleotides were used for the PCR: forward cox-2 primer, 5′-TGCATTCTTTGCCCAGCACT-3′; reverse cox-2 primer, 5′-AAGGCGCAGTTTACGCTGTCT-3′; forward laminin γ2 chain primer, 5′-TGGATGAGTTCAAGCGTACACA-3′; reverse laminin γ2 chain

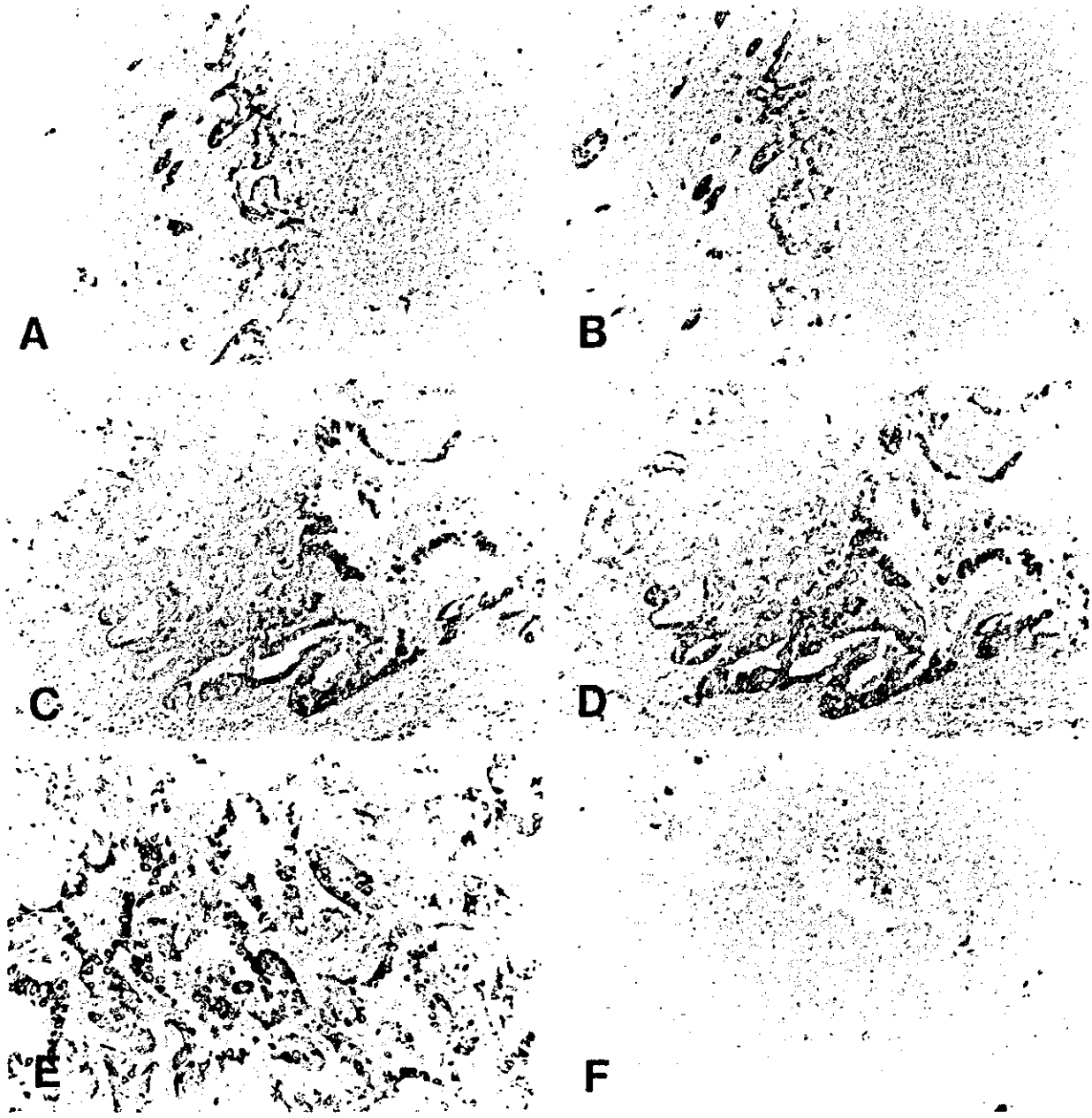

Figure 1. Expression and localization of cox-2 and laminin-5. Cox-2 and laminin-5 were frequently co-localized in the cytoplasm of cancer cells at the cancer-stroma interface. **A:** Cox-2. **B:** Laminin-5. In some cases, cox-2 and laminin-5 were co-localized in cancer cells near the necrotic area. **C:** Cox-2. **D:** Laminin-5. In other cases, discrepancy in distributions occurred owing to a somewhat diffuse staining pattern of cox-2, and to relatively strong cox-2 staining in some bronchioloalveolar carcinomas. **E:** Cox-2. **F:** Laminin-5.

primer, 5′-GCTTTTAGCAAGATTGGCACG-3′. These primers were designed by using the computer program Primer Express (Perkin-Elmer) following the manufacturer's instructions. Primers were chosen from sequences of different exons. Sequence specificity of the primers was confirmed by homology searches through databases at NCBI by using the computer program BLASTN. Primers were purchased from Greiner Japan (Tokyo). To normalize the data, 18S rRNA was quantitated by real-time RT-PCR using the TaqMan Ribosomal RNA Control Reagents kit. After normalization, the results were expressed in arbitrary units. Negative controls lacking template RNA were always included in each experiment.

Western Blot Analysis

Cell lysates were obtained as follows: for total cell lysates, cells were lysed in a lysis buffer consisting of 50 mmol/L Tris-HCl (pH 6.8) and 2% sodium dodecyl sulfate with a cocktail of proteinase inhibitors. After sonication, lysates were boiled for 5 minutes and cleared by centrifugation. For phosphoprotein analysis, cells were lysed in a cold buffer containing 1% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, and a cocktail of proteinase inhibitors under constant shaking for 30 minutes, and then cleared by centrifugation. Protein concentrations were