endothelial cell detachment, which is required for the induction of migration. However, depending on the conditions present and phases, excessive reduction of cell adhesion may inhibit endothelial cell fusion and network formation. In agreement with this notion, we observed impaired network formation of ALK-5-infected HUVEC on Matrigel culture (see Fig. 1C).

Integrin β_5 was downregulated only by ALK-5 (Table 4). Integrin β_5 was isolated as a vitronectin receptor in a human lung epithelial-derived cell line (McLean et al., 1990). Integrins constitute α- and β-subunit heterodimers and function as cell–matrix adhesion molecules, interacting with fibronectin, vitronectin, and collagen. They also mediate intracellular signaling through interaction with cytoplasmic proteins. We also found that the expression of integrin α_E was increased by ALK-1 and that it may have been upregulated by ALK-5, although the intensity of integrin α_E was slightly below 100 in ALK-5-infected HUVEC (Table 1). Integrin α_E was reported to be induced by TGF-β and expressed in mucosal lymphocytes (Shaw et al., 1994). Integrins play major roles in cell adhesion, morphogenesis, migration, and lumen formation in contact with ECM, but it is difficult to determine whether the repression of integrin β_5 by ALK-5, with the induction of integrin α_E, has positive or negative effects on vascular formation.

Other genes possibly involved in vascular function

We extracted about 150 genes, possibly involved in vascular function (Table 5). From among the selected genes, we focused on certain genes, and discuss them below.

VEGF strongly stimulates endothelial cell proliferation and migration and plays essential roles in vascular development (Shalaby et al., 1995; Ferrara et al., 1996). Among three types of VEGF receptors, VEGFR3, which is involved in lymphatic and vascular development (Dumont et al., 1998; Karkkainen et al., 2000), exhibited a significant level of expression (GeneChip intensity of control cells = 249) and was decreased 1.9-fold by ALK-1 (Table 5). However, it was not clearly determined whether the effect of VEGF on endothelial cells is regulated by TGF-β.

The endothelial receptor tyrosine kinases Tie1 and Tie2, and the Tie2 ligands, angiopoietins, are indispensable for vascular remodeling. Tie-2-deficient mice displayed dilated vessels without pericytes or smooth muscle cells, similar to angiopoietin-1-null mice (Sato et al., 1995; Suri et al., 1996). Disruption of the Tie1 gene in mice resulted in systemic hemorrhage, suggesting that Tie1 is also required for vascular stabilization (Sato et al., 1995). Expression of Tie2 was increased from 57 to 136 by ALK-1 and to 129 by ALK-5, although fold changes were less than 2 (Table 5). Previous investigations suggest that effects of angiopoietin-Tie2 signaling on vascular development are similar in certain aspects to those of TGF-β. It is possible that TGF-β regulates activation and resolution of vascular tissues by ALK-1 or ALK-5 through modulation of angiopoietin signals, but further investigation of this question is required.

MMPs play essential roles in vascularization by degrading ECM components, inducing invasion, and activating growth factors sequestered in ECM (Carmeliet, 2000). In our study, we found that both ALK-1 and ALK-5 repressed MMP14/MT1-MMP (Tables 2 and 4), which is a membrane binding-type matrix metalloprotease involved in connective tissue metabolism (Holmbeck et al., 1999). In addition to MT1-MMP, a disintegrin and metalloprotease domain 15 (ADAM15) was downregulated by both ALK-1 and ALK-5 (Tables 2 and 4). ADAM15 is expressed in aortic smooth muscle and vascular endothelium (Herren et al., 1997) and was shown to interact with integrin $\alpha_V\beta_3$ (Nath et al., 1999). MMPs, and other proteases including plasminogen activators (PAs), are required for the initial phase of angiogenesis, but excessive proteolysis may inhibit vessel formation. The repression of MT1-MMP and ADAM15 are consistent with the notion that TGF-βs reduce secretion of proteases, while they enhance ECM accumulation, resulting in stabilization of blood vessels.

TGF-β is a potent inhibitor of endothelial cell growth and an inducer of apoptosis. Although, we observed growth inhibition and apoptosis of HUVEC by ALK-5 in the present study (see Fig. 1A,B), we were not able to determine which genes are involved in the inhibition of the growth of HUVEC (see Table 5). It will be important to examine the expression of genes involved in cell cycle regulation at different time periods.

Quantitative analyses of expression levels of the ALK-5- and ALK-1-induced genes in HUVEC

Expression of the genes induced by ALK-5 or ALK-1 in HUVEC was analyzed by quantitative real-time RT-PCR. Expression profiles of Smad6, Smad7, Id1, Id2, endoglin, and plasminogen activator inhibitor-1 (PAI-1) in ALK-1- or ALK-5-infected cells were essentially similar to those obtained by oligonucleotide microarray and Northern blot analyses (Fig. 4A).

Expression of the same genes in HUVEC after treatment with 5ng/ml of TGF-β3 was also analyzed (Fig. 4B). Smad6, Smad7, Id1, and Id2 were transiently upregulated by TGF-β. Since these genes were induced by ALK-1 but not by ALK-5, TGF-β probably activated ALK-1 and induced these genes in HUVEC. Although endoglin was induced by ALK-1 (Fig. 4A), TGF-β did not significantly induce the expression of endoglin in HUVEC (Fig. 4B). The reason for this observation remains to be elucidated. Since TGF-β binds to ALK-1 with lower affinity than to ALK-5 (Oh et al., 2000), it is possible that activation of ALK-1 in HUVEC by 5 ng/ml of TGF-β3 was not strong enough compared to that induced by the infection of the ALK-1 adenovirus. PAI-1 was induced by ALK-5, but not significantly by TGF-β in HUVEC. High basal expression of PAI-1 without stimulation by TGF-β in HUVEC may be responsible for these results (see below).

We have previously reported detection of targets of TGF-β in HaCaT cells derived from human keratinocytes in the absence and presence of cycloheximide using oligonucleotide microarray (Akiyoshi et al., 2001). We identified 32 genes upregulated and 70 genes downregulated by TGF-β in HaCaT cells. We compared all these genes in HaCaT cells with our results on HUVEC. βIG-H3 and tissue factor pathway inhibitor 2, which were induced by TGF-β in HaCaT cells, were also induced by ALK-5 in HUVEC. The induction of βIG-H3 appeared to be indirect in the analysis of HaCaT cells.

PAI-1, a well-known target of TGF-β (Keeton et al., 1991), was strongly induced by TGF-β in HaCaT cells. In the present study with HUVEC, ALK-5 increased the intensity of PAI-1 from 2477 to 3053, although fold change was only a 1.2-fold increase (Table 5), and TGF-β3 did not significantly induce PAI-1 in HUVEC (see Fig. 4B). Although more genes were downregulated by TGF-β in our study in HaCaT cells, the same genes were not downregulated in the two analyses. This result may be due to the difference in cellular functions between epithelial kerationocytes and vascular endothelial cells, since downregulation of gene expression largely depended on baseline expression levels.

CONCLUSION

Oligonucleotide microarray analysis has enabled us to perform a comprehensive survey of many targets of TGF-β in endothelial cells. This approach revealed a remarkable difference in transcriptional targets and their regulation between ALK-1 and ALK-5. The results are summarized in Figure 5. Inspection of cell–matrix interaction genes suggested that ALK-5 might be more important than ALK-1 in the modulation of ECM through βIG-H3, LTBP1, and possibly through other factors induced by ALK-5. Inhibition of proteases including MT1-MMP and ADAM 15 by both ALK-1 and ALK-5 is consistent with the requirement of TGF-β function for vascular maturation. Downregulation of claudin 5 may be involved in impaired network formation of ALK-5-infected HUVEC on Matrigel culture. Regulation of Ephrin-A1, Ephrin-B3, and Eph-B4 by ALK-1 or ALK-5 probably contributes to TGF-β-mediated vascular development. Interestingly, ALK-5 specifically induced SM22α and other smooth muscle cell-related genes in HUVEC, suggesting that ALK-5 may regulate differentiation of periendothelial cells. Intracellular regulators, including Smads, Id1, Id2, and STAT1, were more strongly affected by ALK-1 than by ALK-5. In particular, the induction of Id genes by ALK-1 probably modulates vascular maturation in association with many other bHLH transcription factors. Although, we did not detect morphological changes of ALK-1-infected HUVEC in the present study, it is possible that ALK-1 may play an important role in regulation of the differentiation of HUVEC in vivo. Further investigations of novel genes identified by this study will provide new clues concerning the mechanisms of vascular development by TGF-β and contribute to therapeutic approaches to vascular diseases.

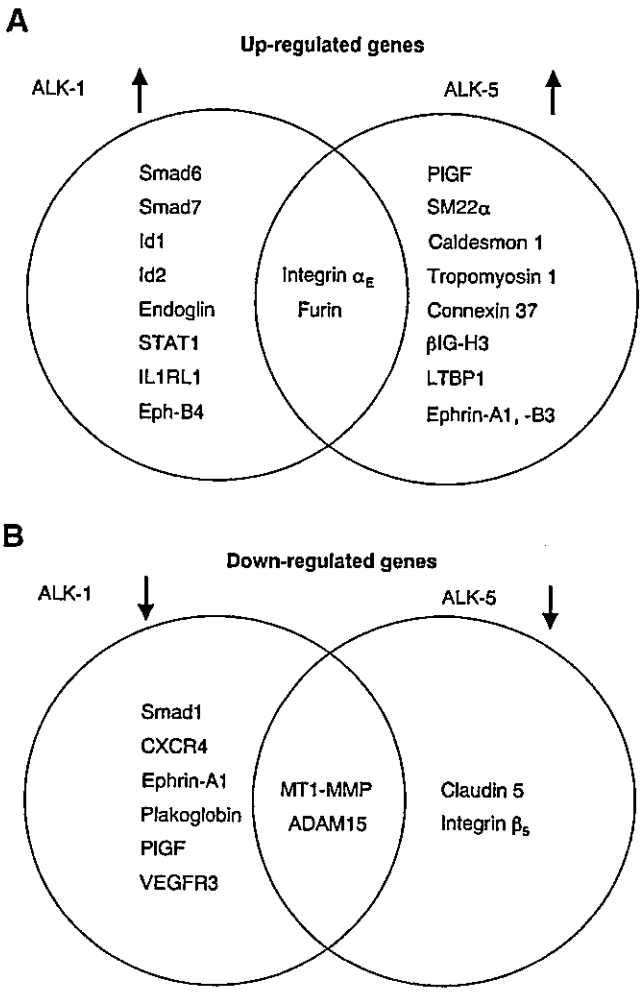

Fig. 5. Transcriptional regulation by activin receptor-like kinase 1 (ALK-1) and/or activin receptor-like kinase 5 (ALK-5) in human umbilical vein endothelial cells (HUVEC). Genes upregulated (A) or downregulated (B) by ALK-1 and/or ALK-5 are shown.

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DETECTION OF AN UP-REGULATION OF A GROUP OF CHEMOKINE GENES IN MURINE CARDIAC ALLOGRAFT IN THE ABSENCE OF INTERFERON-γ BY MEANS OF DNA MICROARRAY[1]

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Backgound. **Interferon (IFN)-γ and the IFN-γ-dependent pathway are prominent in vascularized allograft during acute rejection. However, IFN-γ deficient (IFN-γ−/−) mice can rapidly reject cardiac allografts. To bring the alternative pathway during allograft rejection into more precise focus, we investigated the gene expression profile in murine cardiac allografts in IFN-γ−/− mice by means of DNA microarray.**

Material and Method. **We screened for gene expression changes in murine cardiac allografts of BALB/c H-2^d into both wild-type C57BL/6 H-2^b (n=3) and IFN-γ−/− C57BL/6 H-2^b(IFN-γ−/−, n=4) using Affymetrix oligonucleotide arrays to monitor more than 11,000 genes and expressed sequence tag (ESTs). The heart was heterotopically transplanted. Transplanted hearts were harvested on day 5. As a control, isografts (C57BL/6 to C57BL/6) were also harvested on day 5.**

Results. **On day 5, 64 of the 84 genes induced in the allografts in wild-type mice were not up-regulated in IFN-γ−/− mice. We identified a group of 54 genes that were up-regulated in allografts in IFN-γ−/− mice. Several chemokine genes, including monocyte chemoattractant protein=1 and macrophage inflammatory protein, were induced in the allografts in both wild-type and IFN-γ−/− mice. Interestingly, a group of genes, including C10-like chemokine and platelet factor 4, were specifically induced in the IFN-γ−/− mice.**

Conclusion. **DNA microarray analysis reveals a unique pattern of mRNA expression in allografts in IFN-γ−/− mice as well as a group of genes induced in cardiac allografts in both wild-type and IFN-γ−/− mice, including monocyte chemoattractant protein-1 and monocyte chemoattractant protein-1.**

INTRODUCTION

DNA microarray technology has made it possible to analyze the expression of a large number of genes and revolutionized many areas of biology and medicine (*1*, *2*). Using such a technique, we can obtain many informative insights into various biological mechanisms (*3–5*). We recently identified the gene expression profile in acutely rejected cardiac allografts by means of oligonucleotide array (GeneChip, Affymetrix, Santa Clara, CA) to highlight the genes specifically involved in acute rejection (*6*). Our data indicates that interferon (IFN)-γ signaling plays pivotal role during acute rejection of murine cardiac allografts.

Classical allograft rejection is T cell dependent. In particular, CD4+ T cells are thought to play a critical role through the release of a number of cytokines that in turn stimulate cytotoxic T cells (CTLs), macrophages, and B cells (*7*, *8*). Type I cyokines, i.e., IL-2 and IFN-γ, have been consistently detected in allograft rejection (*9–12*). IFN-γ secreted from the activated T cell, is thought to evoke graft destruction through a stimulation of chemokines and/or an increased expression of class I and class II MHC molecules (*13*). Therefore, it was generally believed that type I cytokines play a central roll in acute rejection.

However, it is also reported that both INF-γ−/− and interleukin-2 (IL-2−/−) mice can acutely reject transplanted heart (*11*, *14–16*). IL-2 and INF-γ double knockout mice can also reject cardiac allografts (*17*). These data suggest that IFN-γ and IL-2 are not absolutely essential for acute rejection by imply a redundant pathway of rejection that is not dependent on INF-γ or IL-2.

IFN-γ has essential immunoregulatory functions in vivo. IFN-γ facilitates the induction of long-term allograft survival and tolerance to protein antigens, partly by limiting the proliferation of activated T lymphocytes, and is an endogenous inhibitor of T cell proliferation and cytotoxic T lymphocyte (CTL) generation in primary mixed lymphocyte reactions (*18*). It also inhibits superantigen-induced clonal expansion of Vb8+ T lymphocytes. Recently, IFN-γ−/− mice

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were shown to be able to rapidly reject cardiac allografts even after depletion of CD4+ T cells indicating a novel CD8+ T cell-mediated mechanism of allograft rejection (*19*). It has also been demonstrated that this pathway of rejection was not suppressable by an anti-CD40 ligand, so it is considered that IFN-γ−/− mice undergo rejection via an alternative pathway. However, the molecular mechanisms underlying novel allograft rejection in the absence of IFN-γ remains unclear.

We report the investigation of the gene expression profile in the rejecting cardiac allograft in IFN-γ−/− mice.

MATERIALS AND METHODS

Mice. Male BALB/c (H-2^d), C57BL/6 (H-2^b) mice were purchased from Clea Japan Inc. (Tokyo, Japan). Male IFN-γ−/− C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Adult males 6–8 weeks of age were used throughout the study. All mice were kept in microisolator cages on a 12-hr day/night cycle and fed on regular food. All procedures involving experimental animals were carried out in accordance with protocols approved in the local institutional guideline for animal care of The University of Tokyo.

Heterotopic cardiac transplant. Cardiac transplants were performed according to the method of Corry and co-workers (*20*). In brief, donors and recipients were anesthetized i.p. before surgery with 4% chloral hydrate at 0.01 ml/g body weight. Donor hearts were perfused with chilled, heparinized saline via the inferior vena cava. The aorta and pulmonary artery of the donor hearts were anastomosed to the abdominal aorta and inferior vena cava of the recipients using a microsurgical technique. The viability of the cardiac allograft was assessed by abdominal palpation and confirmed by observation at laparotomy. Rejection of cardiac grafts was considered complete by the cessation of impulses and confirmed visually after laparotomy.

Histological examination. Heart allografts and isografts were removed from the recipients under anesthesia with 4% chloral hydrate on day 5 after transplantation. The graft was cut transversely into two sections and the basal portion was fixed in 8% paraformardehide with the other section snap-frozen for RNA extraction. The section at the edge of maximal circumference was stained with hematoxylin and eosin.

RNA preparation and Northern blot analysis. On day 5 after heterotopic cardiac transplantation, transplanted hearts were excised from the recipients. Total RNA was then isolated by ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The concentration of total RNA was determined at the optical density of 260 nm. A total of 10-μγ of total RNA were dissolved in 2.2 M formaldehyde, denatured at 65°C for 15 min, and electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde. After transfer to nitrocellulose membranes (Hybond XL, Amersham Pharmacia Biotech, Piscataway, NJ), the filters were hybridized with 2×10^6 cpm of [^{32}P]dCTP-labeled probes per ml.

Microarray analysis. Intragraft gene expression in heterotopic heart grafts was examined via DNA microarray of isolated graft total RNA. The RNA was isolated from 1) nonrejecting cardiac isografts (C57BL/6 to C57BL/6) on day 5 (n=3); 2) rejecting cardiac allografts in wild-type (WT) mice (BALB/c to C57BL/6) on day 5 (n=3); 2) rejecting cardiac allografts in IFN-γ−/− mice (BALB/c to C57BL/6 IFN-γ−/−) on day 5 (n=4). Total RNA was used to generate first-strand cDNA. After second-strand synthesis (GIBCO BRL, Carlsbad, CA), in vitro transcription (Ambion) was performed with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY), resulting in a 40- to 80-fold linear amplification of RNA. Amplified cRNA was purified on an affinity resin column (RNeasy, Qiagen, Tokyo, Japan) and quantitated by spectrophotometer. A total of 40 μγ of biotinylated RNA were fragmented to 50- to 150-nt fragments before overnight hybridization to Affymetrix mouse11K arrays (Mu11KsubA, Mu11KsubB). These arrays contain probe sets for more than 11,000 genes from mRNA transcripts and EST clones. The fragmented cRNA (up to 0.05 μg/μl), control oligonucleotide B2 (up to 50 pM), control cRNA cocktail (5, 25, and 100 pM), acetylated bovine serum albumin (BSA) (to 0.5 mg/ml) and sonicated herring sperm DNA (to 0.1 mg/ml) were added to a hybridization buffer containing 100 mM MES, 1.0 M NaCl, 20 mM EDTA, and 0.01% Tween-20. The hybridization mixture was heated to 99°C for 5 min followed by incubation at 45°C for 5 min before injection of the sample into the probe array cartridge. Hybridizations were carried out at 45°C for 16–17 hr during mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed, the arrays were rinsed with Non-stringent Wash Buffer (0.9 M NaCl, 51.9 mM NaHPO4, 7.5 mM EDTA, 0.01% Tween-20, and 0.005% Antifoam) for 10 cycles of 2 mixes per cycle at 25°C, and the incubated with Stringent Wash Buffer (100 mM MES, 0.1 M NaCl, and 0.01%Tween-20) for 4 cycles of 15 mixes per cycle at 50°C. Hybridized arrays were stained with 5.0 μg/ml streptavidin/phycoerythrin (Molecular Probes, Eugene, OR) and 2.0 mg/ml acetylated BSA (Gibco BRL) in SAPE solution (100 mM MES, 0.1 M NaCl, 0.05% Tween-20, 0.005% Antifoam, 2 μg/ul acetylated BSA and 10 μg/ml SAPE) at 25°C for 10 min. After washes with Non-Stringent Wash Buffer, probe arrays were stained for 10 min at 25°C in antibody solution (100 mM MES, 0.1 M NaCl, 0.05% Tween-20, 2 μg/μl acetylated BSA, 0.1 mg/ml normal goat IgG, and 3 μg/ml biotinylated antibody). The probe arrays were then stained for 10 min in SAPE solution at 25°C. The final wash entailed 15 cycles of 4 mixes per cycle at 30°C with Stringent Wash buffer. Probe arrays were scanned three times at 3 μm resolution using the GeneChip system confocal scanner (Affymetrix, Hewlett-Packard, Santa Clara, CA). Intensity values were scaled such that the overall intensity for each chip of the same type was equivalent (*1*).

Quantitative analysis. The intensity for each feature of the array was captured with GENECHIP SOFTWARE (Affymetrix), and a single raw expression level for each gene was derived from the 20 probe pairs representing each gene by means of a trimmed mean algorithm. A threshold of 20 U was assigned to any gene with a calculated expression level less than 20, because discrimination of expression below this level cannot be performed with confidence (*2*). The expression level of each gene and the fold change between the two experiments was calculated using GeneChip software (Affymetrix). Intensity values were scaled such that the overall intensity for each chip of the same type was equivalent. The average difference of each experiment was normalized to 100. We used a computer program (GeneSpring, Redwood, CA) to cluster and visualize similar gene expression patterns from large sets of data generated by DNA microarray analysis.

RESULTS

The objective of this study was to investigate the alternative pathway during acute rejection in the absence of IFN-γ.

Graft survival. All isografts survived more than 100 days. Mean graft survival times for cardiac allografts in WT mice (n=5) were 8±0.6 days (mean±SEM) and 7.2±0.4 days in

TABLE 1. Survival time of cardiac allograft

Group (strain combinations)	Survival time (days)	Mean survival±SEM (days)
C57BL/6 to C57BL/6	>100, >100, >100, >100	>100
BALB/c to C57BL/6	7,8,8,8,9	8±0.6
BALB/c to C57BL/6 IFN-$\gamma^{-/-}$	7,7,7,7,8	7.2±0.4

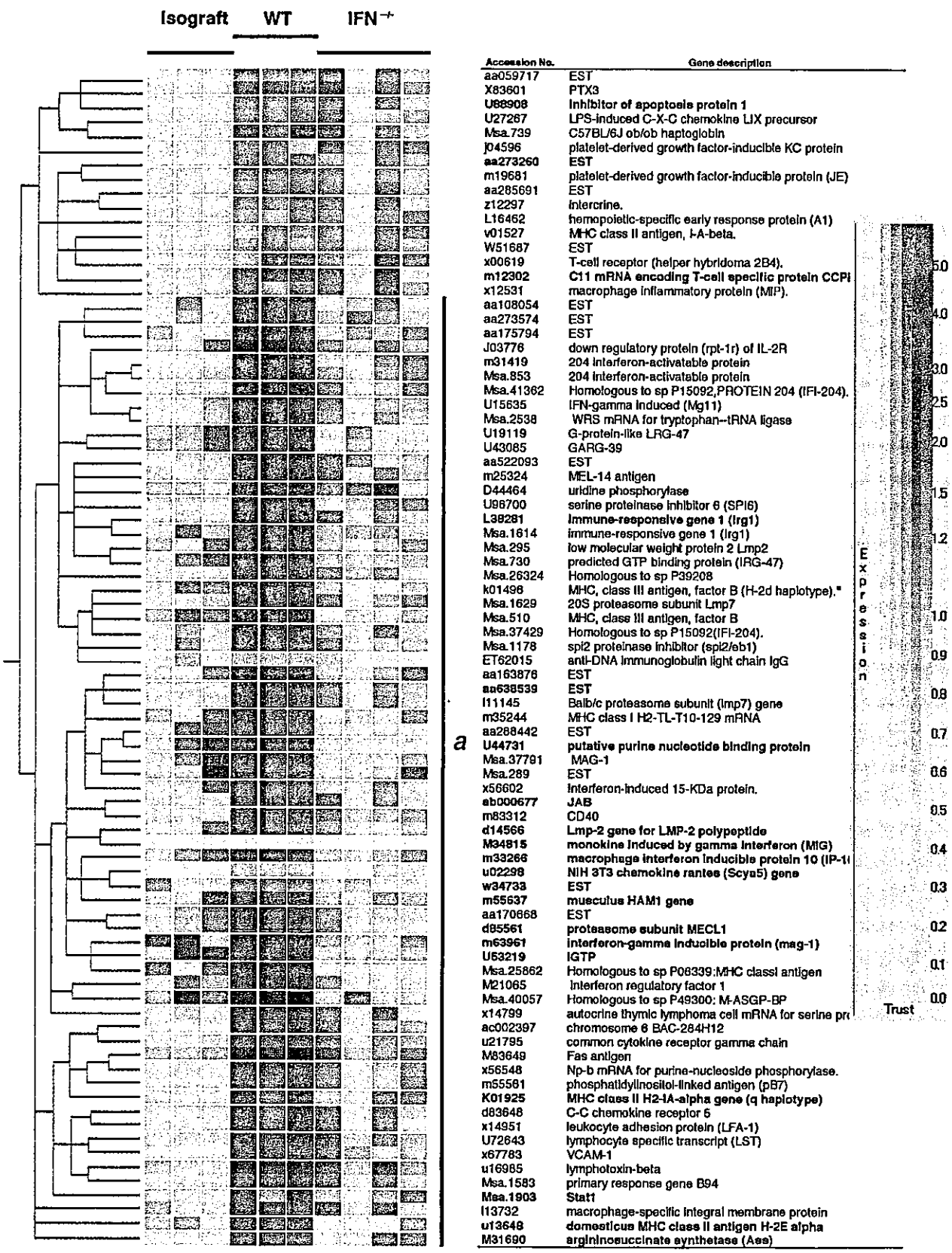

FIGURE 1. Gene expression patterns of a set of 84 genes, which were induced in WT mice rejecting cardiac allografts in day 5 (5). The 84 genes were clustered using a hierarchical clustering computer program (GeneSpring). Data are presented in a matrix format. Each row represents a single gene, and each column an experimental sample. The ratio of the abundance of transcripts of each gene to the median abundance of the gene's transcript is represented by a color in the corresponding sample in the matrix. Blue squares, transcript levels below the median; red squares, transcripts levels above the median. The primary data tables can be viewed at *http://www.med.rcast.u-tokyo.ac.jp/project/transplantation/transplantation.htm*. The gene expression of 68 of these 84 genes was not induced in allografts in IFN-γ−/− mice (a). The top 20 genes profoundly induced in allograts in WT are shown in bold capital letters. Gene descriptions have been edited.

IFN-γ−/− mice (n=5) (Table 1). Thus, there was no significant difference in graft survival between WT mice and IFN-γ−/− mice.

Histological evaluation. On day 5, allografts in wild-type recipients and IFN-γ−/− recipients showed diffuse, perivascular, or interstitial infiltrate of mononuclear cells and some foci of inflammatory infiltrate with myocyte damage. There was no histological difference between allografts in WT and IFN-γ−/− mice on day 5. None of the isografts underwent rejection, as expected for inbred mouse strains.

DNA microarray. Because each sample was hybridized to a separate DNA array, it was essential to determine the consistency of the arrays by calculating the average intensities for all of the GAPDH probes in all the data sets. We observed that the average hybridization signals for each GAPDH probe set differed by less than 50% in each sample.

We screened for gene-expression changes in the isograft and allograft using Affymetrix oligonucleotide expression arrays capable of monitoring more than 11000 genes and expressed sequence tags (ESTs). The data was available on line at *http://www.med.rcast.u-tokyo.ac.jp/project/transplantation/transplantation.htm.*

Genes up-regulated in the allograft in WT mice. The threshold values of an average difference of at least 50.5-fold-change of at least 3.0-fold were considered reliable for genes with expression values significantly over background. The data was analyzed with one-sided *t* tests and adjusted by means of a permutation-style resampling method to control for the false-positive error rate associated the multiple tests. We previously analyzed the trend of gene expressions during acute rejection in WT mice and reported no definitive clusters were specifically induced in the allografts on day 1 or 3 (*6*). Therefore, in this case, we compared the gene expression profile between WT mice and IFN-γ−/− mice on day 5. Previously, we had shown that 84 genes were identified as significant statistically ($P<0.05$) in allografts transplanted in WT mice. The gene expressions of these 84 genes were clustered by computer software (GeneSpring) (Fig. 1). The gene expressions of these 84 genes were not induced in the allografts in IFN-γ−/− mice. The gene expressions in the

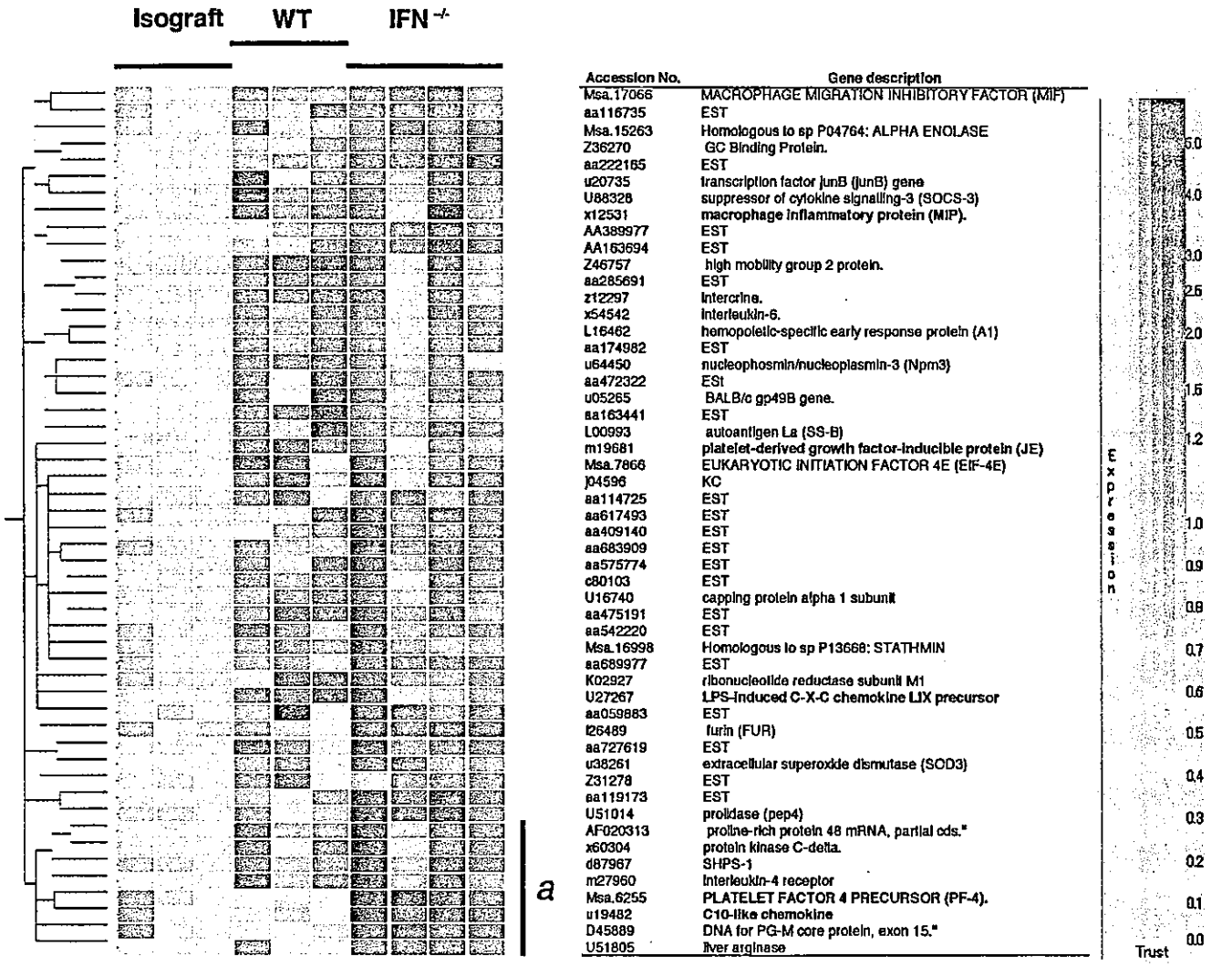

FIGURE 2. Gene expression pattern of a set of 52 genes, which were induced in rejecting cardiac allografts in IFN-γ−/− mice on day 5. Intragraft gene expression in murine cardiac transplantation. The color map indicates a cluster of genes that was specifically induced in allografts in IFN-γ−/− mice (a). Genes of chemokines and chemokine receptors are shown in bold capital letters. Gene descriptions have been edited.

isograft in IFN-γ−/− mice (C57BL/6 to C57BL/6 IFN-γ−/−) showed no certain change in comparison with the gene expressions in the isograft in wild-type mice (data not shown). The gene expressions of 68 of these 84 genes were not induced in the allografts in IFN-γ−/− mice. IFN-γ−/−-inducible genes that play a pivotal role in acutely rejecting allograft transplanted in WT mice, including Mig (monokine induced by IFN-γ−/−), IFN-inducible protein 10 (IP-10) or MHC class I, II antigen, were not involved in allograft rejection in the IFN-IP-10 mice.

Genes up-regulated in allografts in IFN-IP-10 mice. Next, we analyzed the gene expression of the genes profoundly induced in IFN-γ−/− mice. A total of 52 genes were differentially induced in allografts transplanted in IFNγ−/− mice in comparison with isografts. These genes were clustered (Fig. 2). Several chemokines such as JE/MCP-1, MIP-1, platelet factor 4, and the C10-like chemokine were differentially induced in the IFN-γ−/− mice. Interestingly, one cluster consisting of eight genes turned out to be specifically induced in the IFN-γ−/− mice. Platelet factor 4 and the C10-like chemokine were specifically induced in IFN-γ−/− mice. Our data demonstrate the important functional roles of chemokines and/or chemokine receptors in acute rejection. Therefore, we focused on differential gene expression of chemokines and/or chemokine receptors.

Chemokine and chemokine receptor genes in allografts. A total of 27 oligoncleotide probes corresponding to chemokines or chemokine receptors were available on the GeneChip Mu11 k. The color map shows three unique patterns of differential gene expresseion (Fig. 3). Cluster a indicates genes specifically iuduced in allografts in IFN-γ−/− mice. The C10-like chemokine and platelet factor 4 were profoundly and significantly induced only in the allografts in the IFN-γ−/−mice. Cluster b indicates a group of genes which were coexpressed in both WT and IFN-γ−/− mice. It is noteworthy that the genes of JE/MCP-1 and MIP1 were profoundly and significantly induced in both WT and IFN-γ−/− mice. Genes specifically induced in WT are listed in the cluster c. The gene expressions of Mig, IP-10, and RANTES, profoundly induced in allografts in WT mice, were not induced in IFN-γ−/− mice. These results support the idea that IFN-γ−/− mice reject allografts by means of different sets of chemokine or chemokine receptors.

Northern blotting. To further validate the expression changes detected by the DNA microarray, we analyzed differential gene expressions of several cytokine or chemokine in transplanted hearts by Northern blotting (Fig. 4). Several chemokines were analyzed. The data correlated well with that of the DNA microarray. IL-4 was additionally analyzed and no gene expression was detected.

DISCUSSION

This study investigated the gene expression profile in acute rejection in the absence of IFN-γ. Transplanted cardiac allografts were rejected in IFN-γ−/− mice at the same speed in WT mice. Histological analysis showed the same type and degree of rejection. However, the molecular profile of gene expression during acute rejection was dramatically different. These results indicate a novel transcriptional mechanism in acute rejection in the absence of IFN-γ.

An alternative mechanism for acute rejection has been postulated based on the fact that IFN-γ−/− mice can rapidly reject allografts (*11, 15*). Our results indicate that a specific group of chemokines and chemokine receptors contributed to the graft rejection in IFN-γ−/− mice. Gene expression of the IFN-γ−/−-inducible chemokines were not expressed in allograft rejection in IFN-γ−/− mice including Mig, IP-10, and RANTES, although they were prominently induced in acute rejection in WT mice. MCP-1 and MIP-1 were up-regulated during acute rejection in both the WT or IFN-γ−/− mice. In

Accession No.	Gene description	Average difference: Isograft (1st)	Isograft (2nd)	Isograft (3rd)	WT (1st)	WT (2nd)	WT (3rd)	IFN- (1st)	IFN- (2nd)	IFN- (3rd)	IFN- (4th)	A	G
ab000803	CXCR-4	48	159	42	27	37	24	264	75	186	102		
M58004	C10	33	18	15	20	41	38	239	33	149	116		
u19482	C10-like chemokine	297	130	114	203	235	100	1227	367	1114	639		#
Msa.6255	platelet factor 4	234	192	107	82	25	77	1716	648	1333	375		#
l12030	SDF-1-beta	235	298	149	185	147	176	344	254	217	235		
X53798	MIP2	20	51	9	19	22	36	43	19	62	36		
d17630	IL-8receptor	-14	9	5	18	12	18	17	11	1	9		
U27267	LIX	21	37	20	111	109	183	237	65	89	97		#
d83648	CCR5	68	33	43	307	176	374	215	76	214	127	#	
m19681	JE/MCP-1	70	-19	37	1016	1125	923	788	359	743	628	#	#
u50712	MCP-5	170	144	179	565	570	492	301	217	264	259		
j04596	KC	2	-13	27	103	146	79	192	73	141	151	#	
u28493	lymphotactin	5	-5	4	76	49	156	30	65	184	152		
m35590	MIP-1β	-40	-45	0	59	26	5	-17	7	96	-6		
u40672	ectaxin	2	1	6	8	-1	3	17	3	26	7		
U70139	CCR4	31	14	28	82	56	57	53	27	119	34		
x12531	MIP-1	13	13	-9	276	177	233	273	81	650	176	#	#
m33266	IP-10	30	50	65	873	802	353	40	33	43	53	#	
u02298	RANTES	-1	-88	-55	594	535	298	-25	-8	21	12	#	
M34815	MIG	-4	-8	23	1402	1133	1308	-3	-6	-3	-5	#	
u92565	fractalkine	21	-134	-27	134	62	30	-20	44	-3	9		
Msa.1189	P500/TCA3	15	77	41	55	26	53	-14	30	49	23		
Msa.22422	CCR1	83	130	150	139	176	205	60	72	65	58		
m17957	TCA3	-25	-39	-26	-3	-10	-23	-5	-10	17	28		
U88357	TECK	-40	-21	-76	-25	-22	-9	3	-19	-4	-14		
AF016712	AMAC1	-61	-132	-60	-71	-33	-69	-61	-29	-23	-52		
ET62920	CCCKR4	-6	-16	-2	-6	-3	-1	-4	-4	-4	-2		

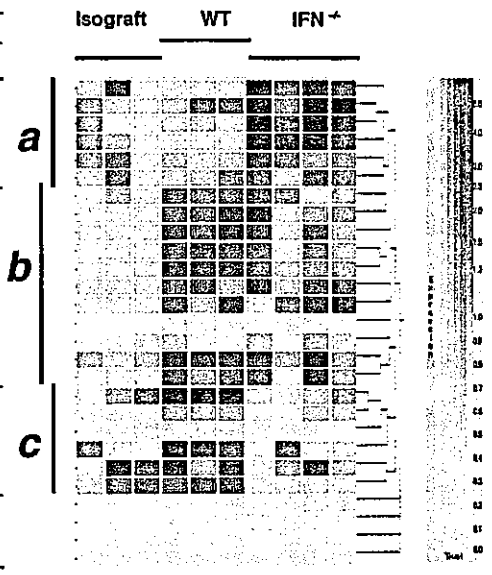

FIGURE 3. **Cluster analysis of chemokine and chemokine receptor genes. The color map indicates three distinct groupings of increased gene expression (a-c). Cluster a, a cluster of genes more intensely expressed in allografts in IFN-γ−/− mice than in WT mice. Cluster b, a cluster of genes expressed in allografts in both IFN-γ−/− mice and WT mice. Cluster c, a cluster of genes that a more intensely expressed in WT mice than in IFN-γ−/− mice. A, Genes significantly induced in allografts in WT mice in comparison with isografts. G, Genes significantly induced in allografts in IFN-γ−/− mice in comparison with isografts.**

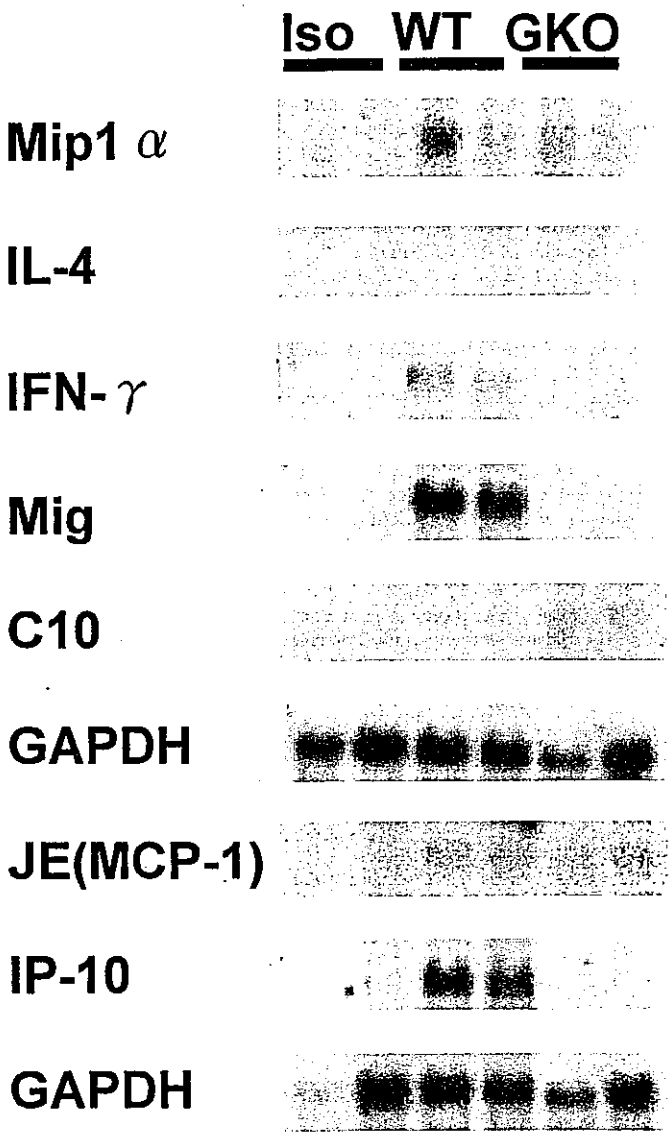

FIGURE 4. **Northern blot analysis. Total RNA extracted from duplicate hearts in each group were fractionated on a agarose gel and then subjected to Northern blotting analysis using α-^{32}P-rediolabeled cDNA probes corresponding to the indicated genes. GAPDH is shown as an internal control. Expression of the mRNA of IFN-γ, Mig, and IP-10 was up-regulated only in the WT mice allografts. Gene expresseion of MIP-1α and C10 were consistently induced in the allografts in both WT and IFN-γ−/− mice. The mRNA of IL-4 was not detected.**

contrast, SDF-1, CXCR4, C10, and platelet factor 4 were up-regulated in IFN-γ−/− mice, although they were not expressed in WT mice. C10 is a novel chemokine expressed in experimental inflammatory demyelinating disorders that promotes the recruitment of macrophages (*21*). MCP-1, MIP-1 and the receptor of SDF-1, CXCR4, have previously been reported to have their transcripts were increased in the rejecting grafts (*22*, *23*). To date, the association between graft rejection and platelet factor 4 or C10 has been very poorly understood. Further studies are needed to clarify this association. Our data show that IFN-γ−/− mice reject cardiac allograft using a unique pattern of transcriptional responses, especially in genes associated with chemokines. As a potent chemotactic factor for monocytes, MCP-1 is thought to be an important chemokine in various inflammatory diseases. MCP-1 is reported to be a potent activator of CD8+CTLs, and CD8+CTLs produce specific chemokines including SDF-1, MIP-1, and MCP-1 (*24*). Recently, Bishop et al. reported the special contribution of CD8+T cell-mediated mechanism in the rejection of cardiac allografts in IFN-γ−/− mice, showing both that IFN-γ−/− mice can reject cardiac allografts in the absence of CD4+ T cells and a special cluster of CD8+ T cells infiltrated in the graft (*19*). In contrast, IFN-γ−/− mice fail to reject MHC class II mismatched skin allografts (*25*). These findings suggest that IFN-γ−/− suppresses CD8+ T cell activity. These data from a DNA microarray reveal the molecular profile of a novel mechanism of rejection. These results support the claim that specific chemokines and CD8+ T cells contribute to acute rejection in IFN-γ−/− mice.

The present data also bear an interesting implication for transplant immunology. In a rodent experimental model, blockade of costimulatory signals has successfully induced tolerance (*26*, *27*). In this study, the gene expressions of LFA-1, VCAM-1, and CD40, which were up-regulated in allografts in WT mice, were not induced in IFN-γ−/− mice. Costimulatory signals do not seem to be associated with allograft rejection in IFN-γ−/− mice. These results support the finding that a blockade of costimulatory signal failed to induce tolerance in an experimental vascularized transplantation model in the absence of IFN-γ−/− (*18*). Most of the current immunogical drugs target Th1 cytokines. The same mchanism reported in this study may be crucial to rejection that is difficult to treat by conventional therapy.

In our study, intraallograft IL-4 mRNA was not elevated on Northern blotting. In the absence of IFN-γ, Th2-cytokines are reported to increase (*15*). IFN-γ−/− mice treated with IL4 mAb did not have an amelioration of graft survival in cardiac transplantation (*19*). Although IL-4 signaling may nevertheless be important in allografs in IFN-γ−/− mice, IL-4, and its subsequent pathway are not essential to rejection in IFN-γ−/− mice. Further study will clarify the critical pathway in acute rejection in IFN-γ−/− mice.

DNA microarrays allow global systematic identification of gene expression on a genome-wide scale. Working with expanded sets of genes, more complete in terms of both number and functional organization, it will be possible to obtain much more information about graft rejection. In this study, we have presented the unique molecular profile of allograft rejection in the absence of IFN-γ, and identified a group of genes induced in cardiac allografts in both WT and IFN-γ−/− mice, including MCP-1 and MIP-1.

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Global Gene Expression Analysis of Gastric Cancer by Oligonucleotide Microarrays[1]

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ABSTRACT

To gain molecular understanding of carcinogenesis, progression, and diversity of gastric cancer, 22 primary human advanced gastric cancer tissues and 8 noncancerous gastric tissues were analyzed by high-density oligonucleotide microarray in this study. Based on expression analysis of approximately 6800 genes, a two-way clustering algorithm successfully distinguished cancer tissues from noncancerous tissues. Subsequently, genes that were differentially expressed in cancer and noncancerous tissues were identified; 162 and 129 genes were highly expressed ($P < 0.05$) >2.5-fold in cancer tissues and noncancerous tissues, respectively. In cancer tissues, genes related to cell cycle, growth factor, cell motility, cell adhesion, and matrix remodeling were highly expressed. In noncancerous tissues, genes related to gastrointestinal-specific function and immune response were highly expressed. Furthermore, we identified several genes associated with lymph node metastasis including *Oct-2* or histological types including *Liver-Intestine Cadherin*. These results provide not only a new molecular basis for understanding biological properties of gastric cancer, but also useful resources for future development of therapeutic targets and diagnostic markers for gastric cancer.

INTRODUCTION

Gastric cancer is one of the leading causes of cancer death in the world (1). Advances in diagnostic and treatment technologies have enabled us to offer excellent long-term survival results for early gastric cancer, but prognosis of advanced gastric cancer still remains poor (2). Recent molecular analyses have clarified many genetic alterations in gastric carcinogenesis, such as *p53* (3), *β-catenin* (4), *E-cadherin* (5), *trefoil factor 1* (6), and c-*met* (7), but this is hardly sufficient to understand common pathway of carcinogenesis and progression of gastric cancer. Furthermore, gastric cancer shows diverse clinical properties such as histological type, metastatic status, invasiveness, and responsiveness to chemotherapy. Little is known about the genes associated with these characteristics.

Gastric cancer tissues generally contain multiple nonepithelial cell types such as fibroblast, smooth muscle cell, endothelial cell, infiltrating lymphocyte, and macrophage. Because recent advances in cancer research have revealed the relevance of epithelial-stromal interaction including ECMs,[3] MMPs, and angiogenic factors in cancer progression (8, 9), we have analyzed whole cancer tissues in this study instead of focusing only on cancer cells to better describe the entire aspect of gastric cancer.

Array technologies are accurate and comprehensive ways of simultaneously analyzing the expression of thousands of genes and have been rapidly applied in many research fields (10). To clarify gene expression changes that are common in cancer tissues or differ among cancer tissues, we have analyzed gastric cancer by oligonucleotide microarray representing approximately 5600 unique genes in this study. We classified both samples and genes by a two-way clustering analysis and identified genes that were differentially expressed between cancer and noncancerous tissues. Furthermore, several genes were identified as being associated with lymph node metastasis or histological types by comparing array data with clinicopathological data.

MATERIALS AND METHODS

Tissue Samples and RNA Preparation. Twenty-six pairs of advanced gastric cancer tissues (T1–T26) and corresponding adjacent noncancerous gastric tissues (N1–N26) were obtained with informed consent from patients who underwent gastrectomy at Jichi Medical College Hospital (Tochigi, Japan). Depth of invasion was more than muscularis propria for all of the cancer tissues, some of which were diagnosed microscopically to accompany lymph node metastasis. A pathologist (J-M. C.) dissected tissue samples from surgical specimens with special care for minimal contamination of nonepithelial cells, and samples were immediately snap-frozen in liquid nitrogen. Another pathologist (H. T.) determined histological classification according to Lauren's classification (11) after H&E staining. These clinical and histopathological features are summarized in Table 1.Tissues were homogenized and lysed directly in Isogen reagent (Nippon Gene, Osaka, Japan). Total RNA was extracted according to the manufacturer's instructions only from tumor specimens that contained >50% cancer cells.

High-density Oligonucleotide Microarray Analysis. Twenty-two gastric cancers (T1–T22) and 8 noncancerous gastric tissues (3N, 4N, 9N, 12N, 16N, and 22N–24N) were analyzed by oligonucleotide microarray (GeneChip HuGeneFL array; Affymetrix, Santa Clara, CA). This array contains 6936 probes interrogating approximately 5600 full-length human genes from the Unigene (Build 18), GenBank, and The Institute for Genomic Research. Analysis was performed essentially as described previously (12). Briefly, double-stranded cDNA was synthesized from 10 μg of total RNA with oligo(dT)$_{24}$ T7 primer, amplified with T7 RNA polymerase up to approximately 100 μg of cRNA, and hybridized to the oligonucleotide microarray according to manufacturer's instructions. For normalization, the average intensity for 6936 genes in total was made equal to 100 to reliably compare variable multiple arrays.

Statistical Analysis. A two-way clustering analysis of 30 samples by Pearson's correlation was performed using the 6272 genes that passed prefiltering, which eliminated genes with an expression level <10 for all of the samples. To identify genes that were differentially expressed between the two groups, Mann-Whitney's U test was used with significance set at $P < 0.05$. Genespring (Silicon Genetics, Redwood City, CA) was used for clustering and statistical analysis. The average expression level of each gene in each group (Ca, cancer tissue; N, noncancerous tissue) was calculated, value below 10 was set to 10, and then the ratio of average expression level between the two groups (Ca:N or N:Ca) was calculated. The cutoff value was set to 80 for average expression level and to 2.5 or 2.0 for the ratio.

Semiquantitative RT-PCR. Single-stranded cDNA was synthesized with oligo(dT) primer in a 20-μl reaction from 5 μg of total RNA using SuperScript

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[3] The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-PCR; LI-cadherin, liver intestine cadherin.

Table 1 *Histopathological characterization of cancer tissue samples*

Histological classification, lymph node metastasis status, and immunoreactivity to anti-p53, E-cadherin, and β-catenin antibodies are described (++, positive for >50%; +, positive for <50%; −, negative). Nuclear staining is regarded positive for p53 and β-catenin, whereas membranous staining is regarded positive for E-cadherin.

Sample	Patient	Histology	Metastasis	p53	E-cadherin	β-Catenin
T1	J-159	Diffuse	−	−	−	+
T2	J-258	Diffuse	+	++	−	−
T3	J-290	Diffuse	+	−	+	−
T4	J-133	Diffuse	+	++	+	−
T5	J-108	Diffuse	+	−	−	+
T6	J-111	Intestinal	+	++	+	++
T7	J-199	Intestinal	+	−	+	−
T8	J-125	Intestinal	+	++	+	++
T9	J-128	Intestinal	−	+	+	+
T10	J-163	Intestinal	+	+	+	−
T11	J-175	Intestinal	+	+	+	−
T12	J-191	Intestinal	−	++	+	−
T13	J-194	Intestinal	+	−	+	−
T14	J-256	Intestinal	−	++	+	−
T15	J-264	Intestinal	−	+	+	++
T16	J-277	Intestinal	−	++	+	++
T17	J-166	Intestinal	+	−	+	+
T18	J-209	Intestinal	−	+	+	+
T19	J-222	Intestinal	+	−	+	−
T20	J-274	Intestinal	+	−	+	−
T21	J-275	Intestinal	+	++	+	−
T22	J-287	Intestinal	+	++	+	−

Preamplification System for First Strand cDNA Synthesis System (Life Technologies, Inc., Rockville, MD) and diluted up to 80 μl. PCR was then performed with 1 μl of cDNA for 1 cycle of 94°C for 2 min, followed by 20–30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min using gene-specific primers and Taq polymerase. Amplification of the right target DNA was confirmed by mobility on gel electrophoresis and sequencing after subcloning into pGEM-T easy vector (Promega, Madison, WI). β-Actin was used as an internal control to confirm equal amount of the templates.

Immunohistochemistry. Formalin-fixed, paraffin-embedded gastric cancer sections were immunostained with horseradish peroxidase-conjugated secondary antibodies using the DAKO LSAB2/HRP kit (DAKO JAPAN, Kyoto, Japan) following the manufacturer's instructions. Primary antihuman antibodies were diluted 1:100 for p53 (DO-7; Novocastra Laboratories Ltd., Newcastle, United Kingdom), 1:1000 for β-catenin (BD; Transduction Laboratories, Lexington, KY), 1:500 for E-cadherin (HECD-1; Takara, Tokyo, Japan), and 1:500 for Oct-2 (C-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

RESULTS

A Two-way Clustering Analysis of Gastric Cancer and Noncancerous Tissues. Twenty-two advanced gastric cancer tissues and 8 noncancerous tissues were analyzed by oligonucleotide microarray, and expression data for 6936 genes were obtained. With expression data of 6272 expressed genes that passed prefiltering, a two-way clustering analysis according to Pearson's correlation was performed. Cancer tissues and noncancerous tissues (30 samples in total) were successfully distinguished (Fig. 1). However, this algorithm using most of the genes on the array did not classify samples among cancer tissues by subgroups associated with histopathological features such as those listed in Table 1.

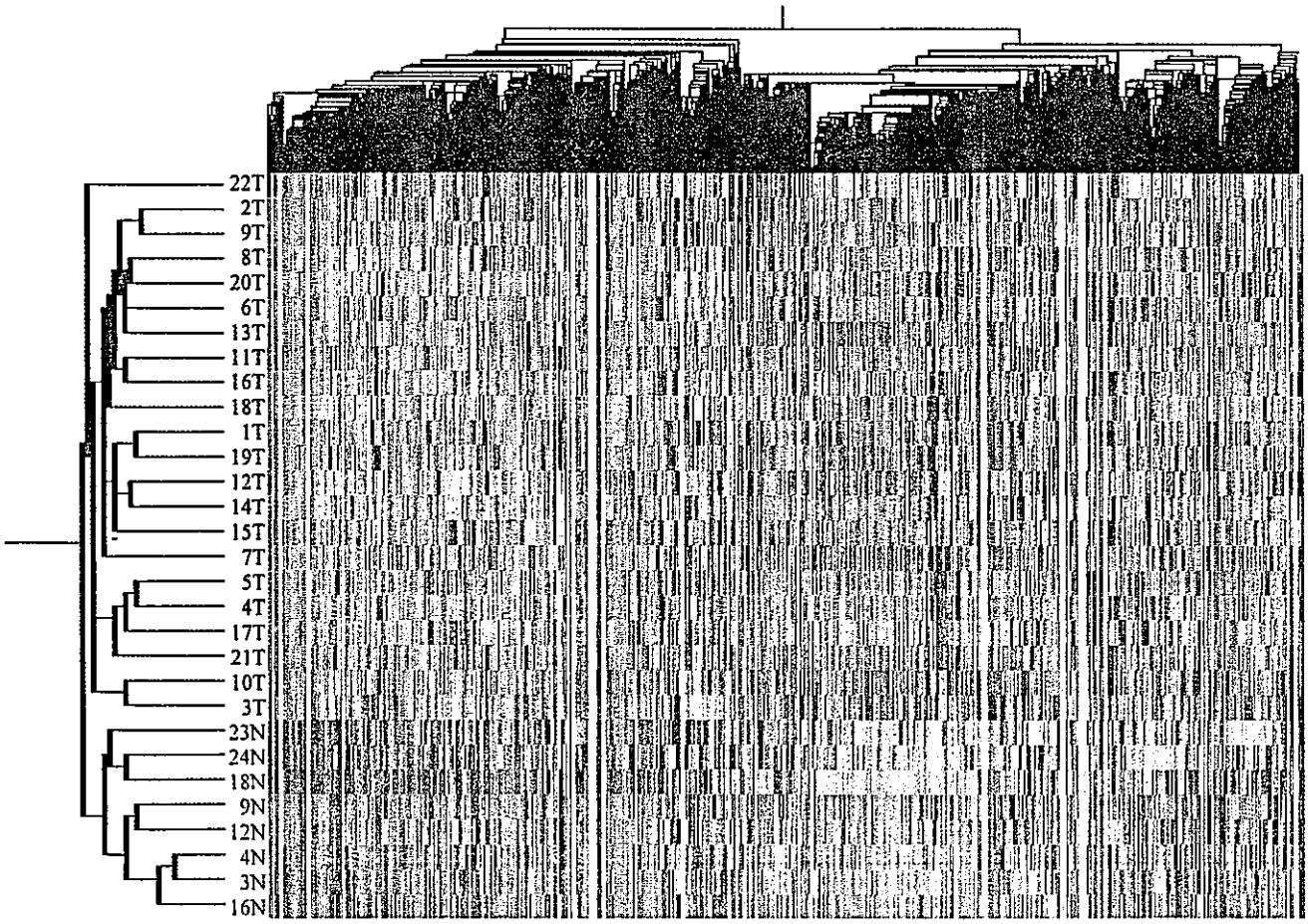

Fig. 1. A two-way clustering analysis of gastric cancer and noncancerous tissues. Thirty samples are lined up on the *vertical axis*, and 6272 genes are in the *horizontal axis*. Cancer tissues (*T*) and noncancerous tissues (*N*) were distinguished. Expression level was normalized per gene, and the relative value to the median among 30 samples is shown by color: *red*; relatively high expression, *blue*; relatively low expression.

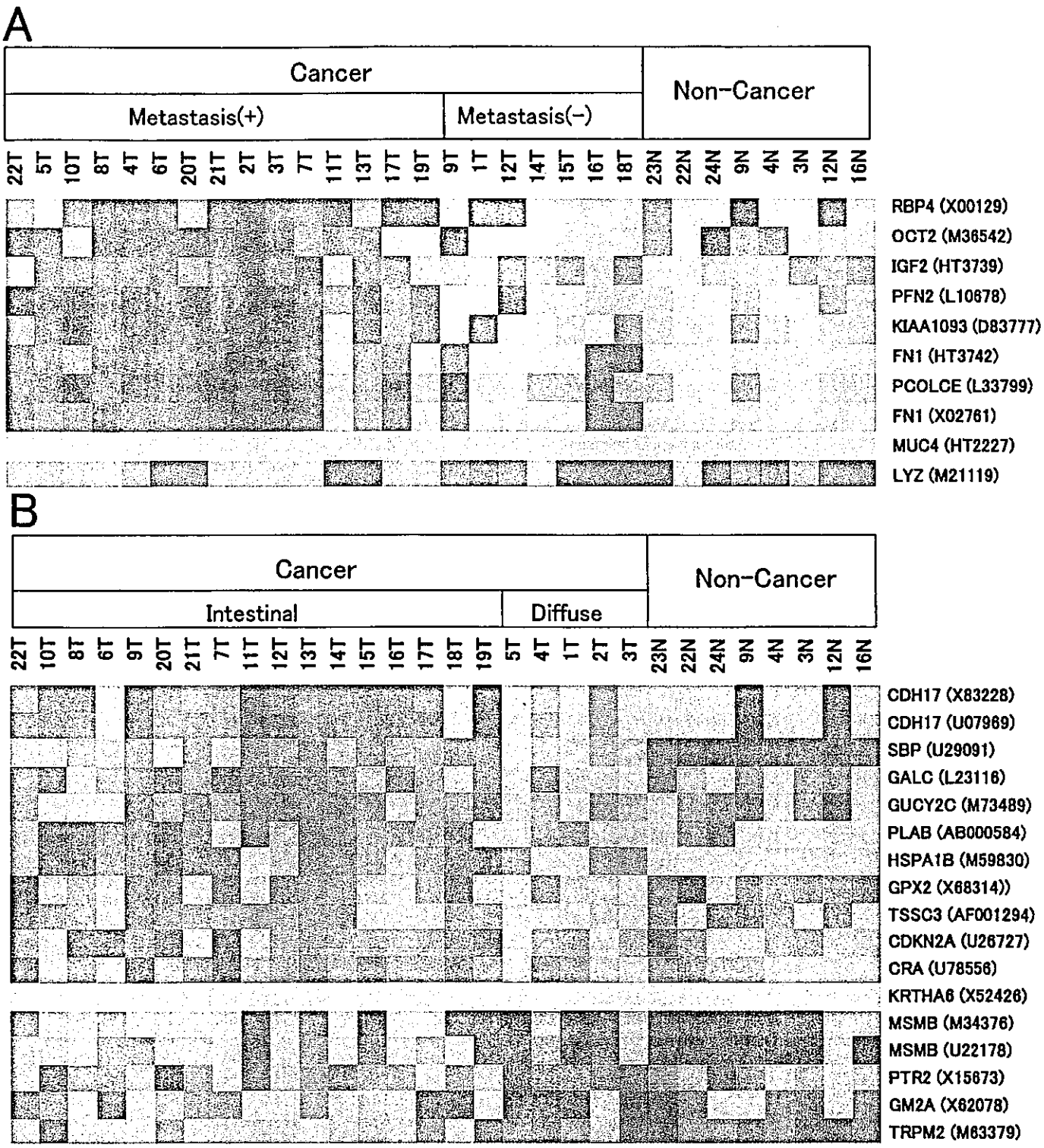

Fig. 2. Genes associated with clinicopathological properties of gastric cancer. Expression level was normalized per gene, and the relative value to the median among 30 samples is shown by color: *red*; relatively high expression, *blue*; relatively low expression. Cutoff value was set to 80 for average intensity and to 2.0 for ratio. GenBank accession numbers are given in *parentheses*. *A*, genes associated with lymph node metastasis. Genes with exclusively high or low expression in metastasis-positive cancer tissues are shown. *B*, genes associated with histological classification. Genes differentially expressed between intestinal-type and diffuse-type cancer are shown. Noncancerous tissue samples are also shown, but only for reference.

Highly Expressed Genes in Gastric Cancer and Noncancerous Tissues. To identify genes that were differentially expressed between cancer and noncancerous tissues, we applied Mann-Whitney's U test to the raw data obtained by microarray analysis. When cutoff values were set to 2.5 for the ratio and 80 for the average expression level to extract only reliable data, 162 and 129 genes were identified that showed higher expression in cancer tissues and noncancerous tissues, respectively ($P < 0.05$). To verify the reproducibility of these gene lists, we performed semiquantitative RT-PCR using the same RNA used for microarray analysis. Four randomly selected pairs of cancer and corresponding noncancerous tissue samples were analyzed in 80 randomly selected genes. Concordant results were obtained for four

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).

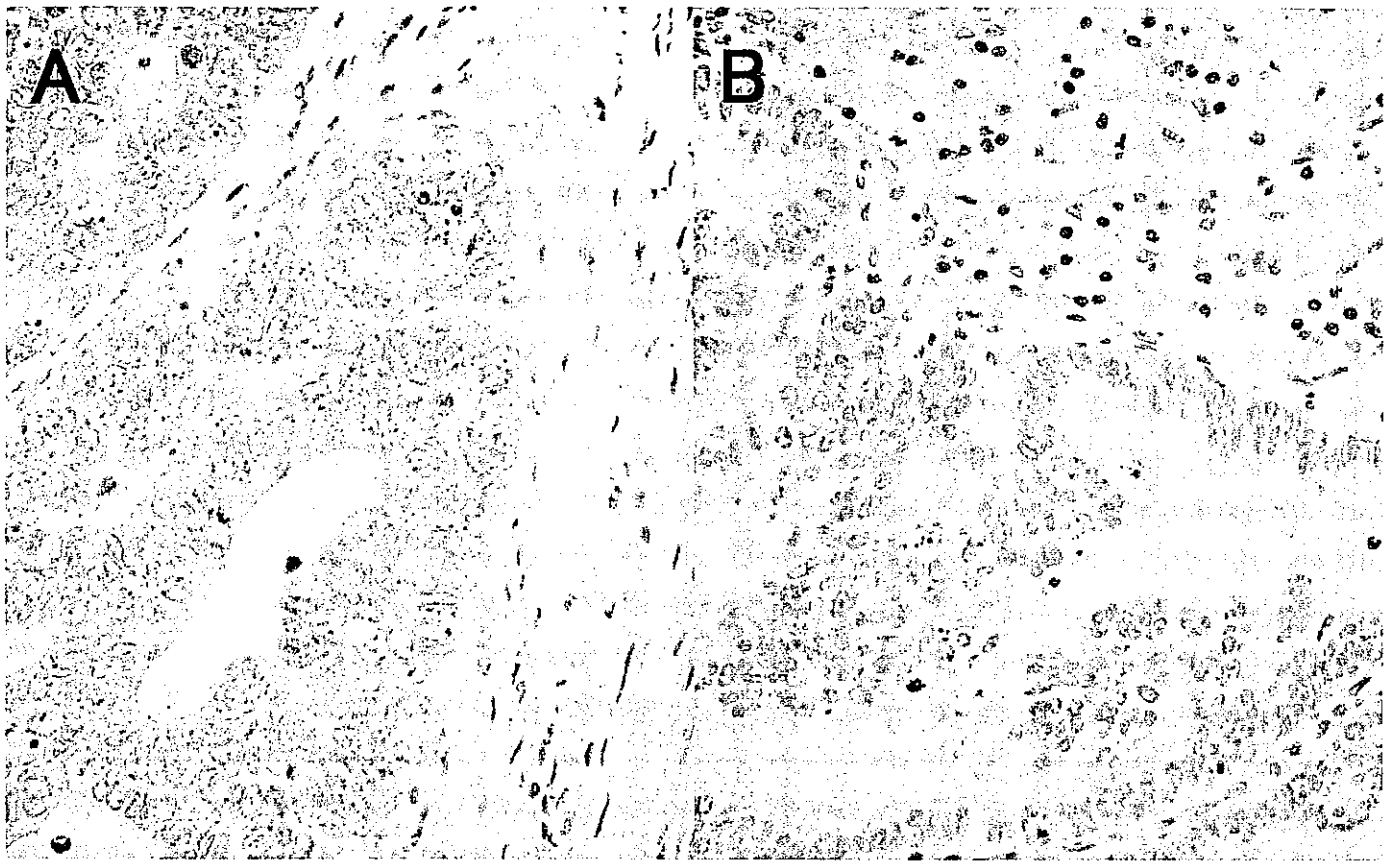

Fig. 3. Immunohistochemical analysis of *Oct-2*. *A*, metastasis-positive cancer tissue (T8). Gastric cancer cells showed strong immunoreactivity. *B*, metastasis-negative cancer tissue (T12). No immunoreactivity was observed for cancer cells. Note that some infiltrating lymphocytes are immunostained 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.

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AN INTEGRATED RECEPTOR DATABASE (IRDB)

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ABSTRACT

Various receptor data were collected, edited and integrated into an Integrated Receptor Database (IRDB). The data stored includes structural data (amino acid sequences, their secondary-structure and three-dimensional structure), functional data, binding affinity, cell signaling data etc. The purpose of this database is to allow structural biologists, drug designers and toxicologists to analyse and elucidate receptor-ligand dockings and the resultant post-binding signal transduction pathways. IRDB is available on line (http://impact.nihs.go.jp/RDB.html)

Keywords: Receptor, Structure, Binding affinity, Cell Signaling, Drug

1 INTRODUCTION

The receptor-ligand binding triggers a series of reactions in a living system. So, detailed knowledge and data about receptors and their ligands are an important basis for understanding living systems and diseases, and for designing new drugs. Because of the advances in molecular biology, which were accelerated by the Human Genome Projects, a huge amount of DNA sequence and protein structural data have been accumulated and are available for public use. Although we had already developed a Receptor Database (Nakata, Takai & Kaminuma, 1999), we have now revised the RDB, integrating new functions and updating the data. The present version of RDB, which we call an Integrated Receptor Database (IRDB), is the updated version of our old Receptor Database (Nakata, Takai-Igarashi, Nakano & Kaminuma, 2001a).

The Internet/World Wide Web (WWW) technology had allowed us to use powerful viewers for representing retrieved data and knowledge graphically. This technology has also allowed us to link RDB dynamically to other related WWW sites. In the previous version of RDB, the goal of our system was to provide one-stop shopping on receptor data. The system uses a good viewer to represent information useful for endocrine disruptor and the drug design; information such as the structural data and binding sites, and the cell signaling pathway that is triggered by a ligand binding. IRDB includes more structural data, binding affinities, the transcription factors and regions, and single nucleotide polymorphisms (SNPs).

2 SYSTEM AND METHODS

2.1 Purpose of the database system

The purpose of RDB is to store data and knowledge on receptor proteins and properties. This data and knowledge includes protein structures and their functions, ligands and binding affinity data, cell signaling information, drug and SNPs. The database users are those who study biology and the mechanisms of disease, and those who are developing drugs based on the structure-based drug design (SBDD) approach and personalized medicine.

2.2 Hardware and software