Breast Cancer
Vol. 9 No. 1 January 2002

Original Article

Analysis of Gene Expression Involved in Brain Metastasis from Breast Cancer Using cDNA Microarray

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Background: Brain metastases occur in 15% to 30% of breast cancer patients, usually as a late event. The patterns of metastases to different organs are determined by the tumor cell phenotype and interactions between the tumor cells and the organ environment.

Methods: We investigated the gene expression profile occurring in brain metastases from a breast cancer cell line. We used cDNA microarrays to compare patterns of gene expression between the mouse breast cancer cell line Jyg MC (A) and a subline that often metastasis to brain, (B).

Results: By Microarray analysis about 350 of 21,000 genes were significantly up-regulated in Jyg MC (B). Many candidate genes that may be associated with the establishment of brain metastasis from breast cancer were included. Interestingly, we found that the expression of astrocyte derived cytokine receptors (IL-6 receptor, TGF-beta receptor and IGF receptor) were significantly increased in Jyg MC (B) cells. These results were confirmed by RT-PCR.

Conclusion: These results suggest that cytokines produced by glial cells *in vivo* may contribute, in a paracrine manner, to the development of brain metastases from breast cancer cells.

Breast Cancer 9:26-32, 2002.

Key words: cDNA Microarray, Brain metastasis, Breast cancer, Cell line, Cytokine

Metastasis formation results when specialized tumor cells ("seeds") find a suitable environment ("soil") for arrest, invasion, and growth[1]. The patterns of metastasis to different organs are determined by the tumor cell phenotype (i.e., the potential for invasion, adhesion, and growth) and interactions between the tumor cell and the organ environment, such as cellular components, cytokines, or organ-derived growth factors[2]. Breast cancer is one of the most common sources of brain metastases. After clinical detection of such tumor spread, median survival is usually only a few months, with brain metastases being the major cause of death[3].

The clinical failure in dealing with breast cancer is due in part to the ability of these carcinoma cells to develop metastatic tumors in distant organs, at locations independent of the vascular anatomy[5]. Common sites of breast cancer metastasis is axillary lymph nodes, bone, lung, liver, and brain. Brain metastasis occurs in 15% to 30% of breast cancer patients[4-6], often presenting as a late event[7]. Thus, the brain environment might play a specific role in the establishment and growth of metastatic breast cancer cells that reach the brain through the blood-brain barrier. The mechanism underlying brain metastasis is unknown.

We would like to elucidate the gene expression profile causing brain metastasis. We used mouse cDNA microarrays to comprehensively analyze gene expression in abundance of approximately 21,000 distinct transcripts from a highly brain metastatic cell line. Variants of the mouse breast cancer cell lines Jyg MC (A) and its highly brain metastatic subline Jyg MC (B) have been described. It has been demonstrated that Jyg MC (B) had different metastatic abilities when grown in the mammary fatpad of nude mice. The highly metastatic Jyg MC (B) cells were originally established from a brain

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Received May 25, 2001; accepted September 14, 2001

Table 1. Incidence of Metastases in Several Organs of Nude Mice Inoculated with Jyg MC (A) and (B)(Morimoto, *et al.* 1995)

	lung	liver	kidney	lymph	heart	spleen	adrenal	brain
Jyg MC (A)	100	40	40	20	60	0	20	0
Jyg MC (B)	100	60	60	20	60	20	20	100

The only difference is in the incidence of brain metastasis between Jyg MC (A) and (B).

metastasis in a nude mouse but did not differ in invasive potential or growth factor responsiveness *in vitro* when compared with the heterogeneous parental cell line[8].

Materials and Methods

Cell Culture

Two sublines of breast cancer cells that were established from the same mammary epithelium were provided from Riken Gene Bank. One was Jyg MC (A) and the other was Jyg MC (B), which differ only in the potential for brain metastasis as shown as Table 1[8]. The Jyg MC (A) and (B) samples used were passage 7-10 cultures that were harvested at 60-80% confluence. The medium changed was 2 days before the mRNA harvest.

Preparation of mRNA from Cells

Cell lines were grown from NCI DTP frozen stocks in RPMI-1640 supplemented with phenol red, glutamine (2 mM) and 10% fetal bovine serum. To minimize the effects of variations in culture conditions or cell density on differential gene expression, we grew each cell line to 80% confluence and isolated mRNA 24 hours after transfer to fresh medium. The time between removal from the incubator and lysis of the cells in RNA stabilization buffer was minimized (< 1 min). Cultured cells were harvested by scraping and Poly (A)+ RNA was prepared using a micro-MACS RNA purification kit and protocol (Miltenyi Biotec. CA. USA).

Preparation of Target DNAs

The target DNAs were collected from RIKEN mouse cDNA libraries[9], which were constructed by using the CAP trapper method to enrich for full-length inserts. The cDNAs were amplified using M13 forward and reverse primers in a 100-μl PCR with 0.2 μM final concentrations of forward (F1224, 5′-CGCCAGGGTTTTCCCAGTCACGA-3′) and reveprse (R1233, 5′-AGCGGATAACAATTTCACACAGGA-3′) primers, 250 μM dNTPs, and 1.25 units of Ex *Taq* in 1 × Ex *Taq* buffer (Takara Shuzo, Tokyo). The PCR product was precipitated with isopropyl alcohol and resuspended in 15 μl of 3 × SSC. The DNA solution was spotted on poly(L-lysine)-coated slides by using a DNA arrayer (http://cmgm.stanford.edu/pbrown/mguide/index.html) with 16 tips (SMP3, TeleChem International, Sunnyvale, CA). The diameter of the spots was 100-150 μm. Mouse -actin and glyceraldehyde-3-phosphate dehydrogenase cDNAs were used as positive controls and *Arabidopsis* cDNAs were used as negative controls (accession nos. X98108, X13611, X90769, Z99707, AF004393, Z49777, Q03943, U58284).

Performance of Riken Microarrays

Many of the cDNAs used to prepare target DNAs were full-length and relatively long. For this reason, we were concerned about the possibility that probes from multiple related transcripts might bind to single targets. The signal intensity of a clone that was about 80% identical to the target sequence was one-tenth that of a completely identical clone. Clones that were less than 80% identical to the target sequence gave signals at the background level (data not shown).

Preparation of the Probe

One microgram of mRNA extracted from Jyg MC (B) cells were labeled by incorporating Cy3 during random-primed reverse transcription. cDNA derived from entire Jyg MC (A) cells, which we labeled with Cy5, was used as the expression reference. Deoxynucleotides labeled with the dyes Cy3 and Cy5 were obtained from Amersham Pharmacia. The labeling was carried out at 42℃ for 1h in a total volume of 30 μl containing 400 units of Super-Script II (GIBCO/BRL), 0.1 mM Cy3-dUTP (or Cy5-dUTP), 0.5 mM each dATP, dCTP, and dGTP, 0.2 mM dTTP, 10 mM DTT, 6 μl of 5 × first-strand buffer, and 6 μg of random primers. To remove unincorporated nucleotides, labeled cDNA was

mixed with 500 μl of binding buffer (5 M guanidine thiocyanate/10 mM Tris-HCl, pH 7.0/0.1 mM EDTA containing 0.03% gelatin and 2 ng/μl tRNA) and 50 μl of silica matrix buffer (10% matrix/3.5 M guanidine hydrochloride/20% glycerol/0.1 mM EDTA/200 mM NaOAc, pH 4.8-5.0), transferred to a GFX column (Amersham Pharmacia), and centrifuged at 15,000 rpm in a Sorvall centrifuge (RC-3B plus; H6000A/HBB6 rotor) for 30s. The flow-through was discarded, and the column was washed with 500 μl of wash buffer. The adsorbed probe was eluted into a final volume of 17 μl of distilled water. This labeled probe was mixed with blocking solution containing 3 μl of 10 μg/μl oligo (dA), 3 μl of 20 μg/μl yeast tRNA, 1 μl of 20 μg/μl mouse Cot1 DNA, 5.1 μl of 20 × SSC, and 0.9 μl 10% SDS.

Array Hybridization and Data Analysis

The RIKEN full-length mouse cDNA that comprised the target was hybridized in a final volume of 30 μl; the entire array consisted of three multiblocks, and each multiblock required 10 μl of hybridization solution. Before hybridization, probe aliquots were heated at 95℃ for 1 min and cooled at room temperature. Coverslips were hybridized overnight at 65℃ in a Hybricasette (obtained from ArrayIt.com). After hybridization, slides were washed in 2 × SSC/0.1% SDS until the coverslips dropped off, and the slides were then transferred into 1 × SSC, shaken gently for 2 min, and rinsed with 0.1 × SSC for 2 min. After washing, slides were spun at 800 rpm in a Sorvall centrifuge (RC-3B plus; H6000A/HBB6 rotor). These slides were scanned on a ScanArray 5000 confocal laser scanner, and the images were analyzed by a ScanAlyze 2 (M.B.E.: http://www.microarrays.org/software.html)[11].

Analysis of the Data

To improve the accuracy of the data, we did the experiments were performed twice, labeling the same RNA template in two separate reactions. Data were normalized to the reference standard by subtracting (in log space) the median observed value if it was other than zero. We used only data points that were reproducible. To this end, we developed a filtering program, PRIM (Preprocessing Implementation for Microarray)[10]. Briefly, this program (*i*) deletes the results with "flags" added manually to corrupted spots, (*ii*) eliminates spots with signal intensities less than the mean +3 × standard deviations of the background signal intensity in either Cy3 or Cy5, and (*iii*) eliminates spots located outside the least-mean-squares line ±2 × standard deviation. After the filtering was finished, we compared the results of the two experiments by calculating Pearson's correlation coefficient.

RT-PCR

As an independent test, we measured the expression levels of several genes using reverse transcription polymerase chain reaction (RT-PCR) of mRNA. For RT-PCR analysis, we used superscript II kit (GIBCO-BRL) and 5 units of Ex *Taq* (TAKARA Inc.) and 0.5 μM of primers. We designed primers as follows: IL-6 receptor (5′-CAGGCAATGTTACCATTCAC-3′, 5′-GTGAGGAGAGGAACCAGAAG-3′), IGF-1 receptor (5′-GCAGACCTCTGACAAGGATG-3′, 5′-CAAGCCAGGTCAACTCTACA-3′) and beta-actin (5′-GATCATGTTTGAGACCTTCAAC-3′, 5′-AATGATCTTGATCTTCATGGTG-3′). PCR products were loaded and electrophoresed on 1% agarose gels at 100 V for 1 hour as described previously.

Results

The cDNA Microarray Analysis

The cDNA made from purified mRNA from Jyg MC (B) was labeled with the fluorescent dye Cy3. A reference probe consisting of cDNA made from purified mRNA from Jyg MC (A) was labeled with a second fluorescent dye, Cy5. The color images of the hybridization results (Fig 1) were made by representing the Cy3 fluorescent image as red and the Cy5 fluorescent image as green and merging the two color images. The throughputs of the normalization and filtering program are shown in Fig 2. Two gene expression profiles of interest, Interleukin 6 receptor and Insulin like growth factor 1 receptor are shown. These results demonstrate that the astrocyte-derived cytokines stimulate the growth of brain metastatic cancer cells as described in the discussion.

Analysis of Gene Expression in Mouse Breast Cancer Cell Lines

We studied gene expression profiles in the cell lines using DNA microarrays prepared by robotically spotting 17,000 mouse cDNAs on glass microscope slides. The cDNAs included approximately 8,000 different genes; approximately 3,700 represented previously characterized mouse proteins, an additional 1,900 had homologues in other organisms and the remaining 2,400 were identified only

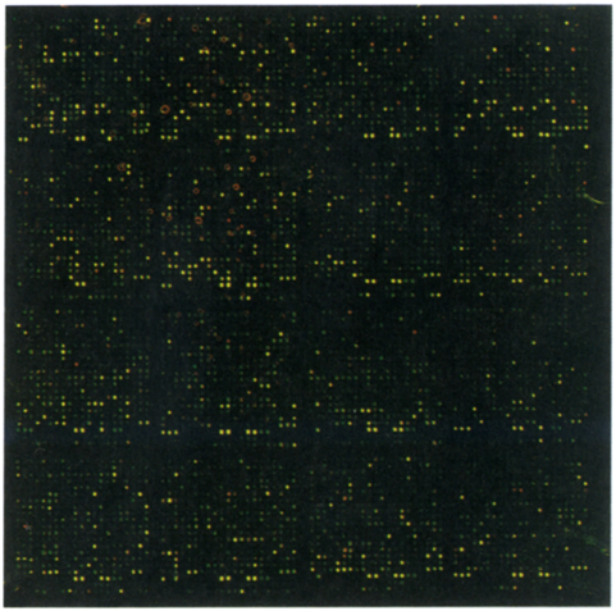

Fig 1. cDNA microarray scanning image. Red is the Cy3 fluorescent image, up-regulated in Jyg MC (B). Green is the Cy5 fluorescent image, up-regulated in Jyg MC (A). Yellow means the same expression level in the two cells.

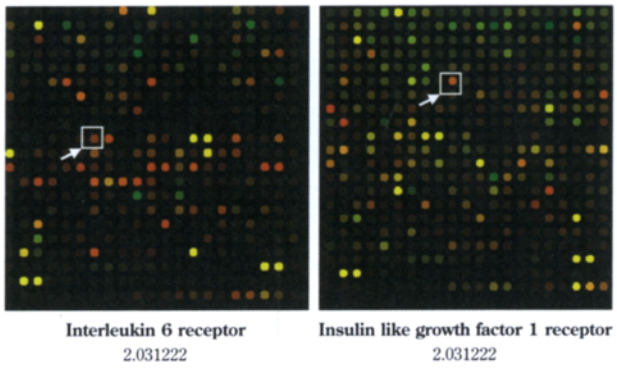

Fig 2. Pseudo color imaging of Interleukin 6 receptor and Insulin like growth factor 1 receptor expression pattern. About 350 genes were significantly up-regulated in Jyg MC (B). After functional categorization, we found up-regulated genes of interest, including astrocyte derived cytokine receptors.

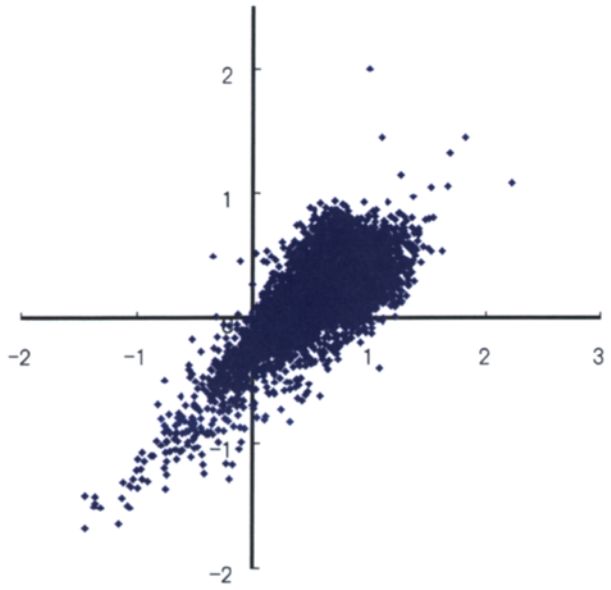

Fig 3. Graphs of two independent hybridizations. Correlation was up to 0.80. This study is confirmable.

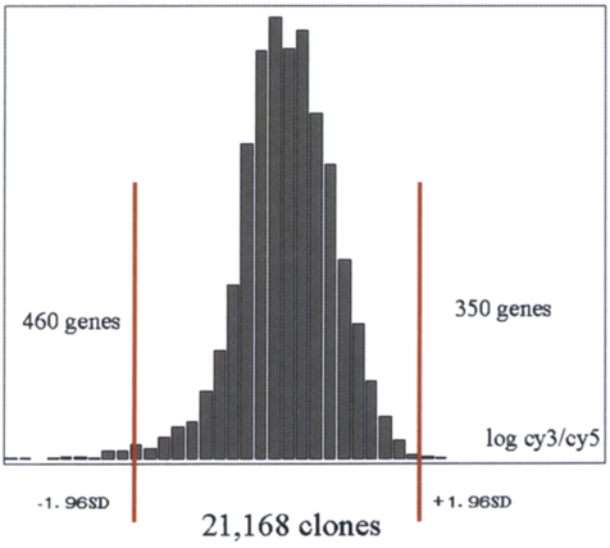

Fig 4. Histogram of microarray analyzed data. About 18,000 of 21,168 genes were throughput from the filtering program. Bar is ± 1.96 SD.

by ESTs. Due to ambiguity of the identity of the cDNA clones used in these studies, we estimated that approximately 80% of the genes in these experiments were correctly identified. The identities of approximately 3,000 cDNAs from these experiments have been sequence- varified, including all of those referred to here by name. Each hybridization compared Cy3-labelled cDNA reverse transcribed from mRNA isolated from Jyg MC (B) with Cy5-labelled cDNA reverse transcribed from mRNA isolated from Jyg MC (A). An additional check was provided by the inclusion of two independent duplicate hybridizations to different microarrays with mRNA samples from cells, which showed good correlation (up to 0.80) (for graphs and details, see Fig 3). The variance in the two fluorescence ratio measurements approached a minimum when the fluorescence signal dynamic range was above background in either channel of the hybridizations. We selected the subset of spots for which significant

Down regulated genes

-3.47114	transferrin:mammary gland
-2.88417	transferrin:mammary gland
-2.69430	CDPK II mRNA
-2.62152	Myc antagonist Mnt
-2.56045	ets2 protein
-2.32711	type XV collagen
-2.32342	TROP 2 mRNA
-2.26300	collagen alpha 1 type II
-2.23986	zinc finger transcription factor
-2.23258	NADH-cytochrome b5 reductase
-2.04841	JAC3
-1.89493	Csk: brain: mus musculus
-1.82759	Ets transcription factor Spi-B
-1.82714	s-CoA d mRNA
-1.78178	GAPDH mRNA
-1.73373	inhibitor of apoptosis homolog
-1.56357	mutant p53 binding protein 1
-1.56009	OSF-1
-1.47891	Smad5
-1.43042	Cdc6-related protein
-1.27279	FK506 binding protein
-1.09930	Cdc25M2
-1.09827	beta-tubulin

Up regulated genes

2.031222	interlukin-6 receptor
1.967690	telomerase RNA
1.927040	preprocathepsin B
1.907171	interleukin-3 gene
1.751838	cyclin D1
1.744325	mouse insulin like growth factor receptor 1 gene
1.744325	Smad 6 mRNA
1.694488	retinol-binding protein
1.693961	heat shock protein
1.639042	cyclin D1
1.605082	brain acyl-CoA synthtase II
1.570351	NADP-dependent isocitrate dehydrogenase
1.565061	nucleic acid binding protein
1.563413	FK506 binding protein
1.546967	tyrosinase
1.534662	ATP synthase beta
1.515628	protocadherin
1.482535	polyubiquitin
1.447304	transcription factor BTF3
1.406794	heparin-binding EGF-like growth factor
1.405466	6-phosphogluconate dehydrogenase
1.400844	interferon-g induced GTPase
1.360085	cyclin G
1.100276	transforming growth factor-beta
1.090753	PDGF associated protein
1.063426	ribosomal protein
1.057979	epididymal secretory protein

Fig 5. Analysis data of cDNA microarray (extracted). About 450 genes were down regulated and 350 genes were up-regulated in Jyg MC (B). To compare the differences of gene expression of the 2 cell lines, we extracted named genes.

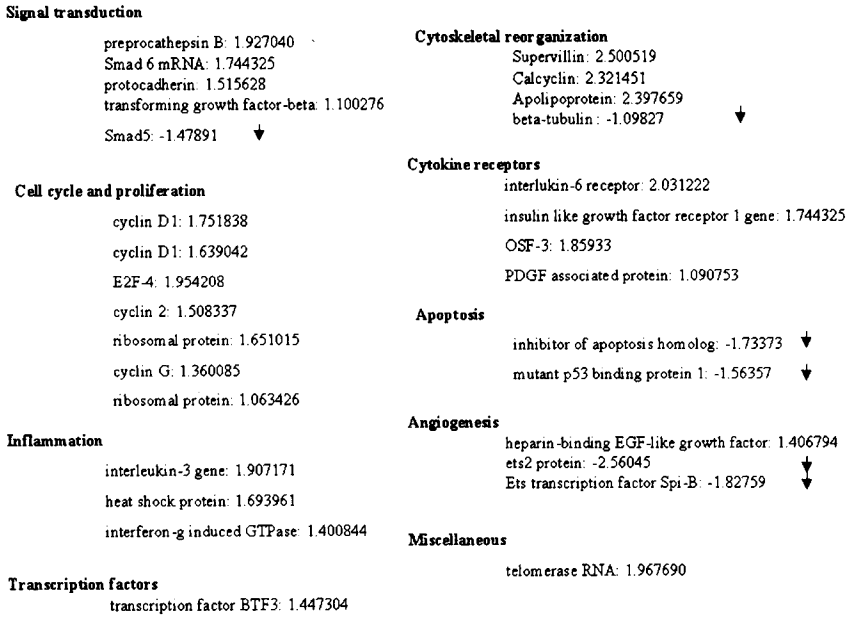

Fig 6. Categorization of gene function. Based on the microarray data, we divided the gene list into a functional category. We could see differences in astrocyte derived cytokine receptor expression between Jyg MC (A) and (B), which may play an important role in the evolution of brain metastasis.

signal was present in both the numerator and denominator of the ratios by this criterion to identify the best-measured spots. The microarray data became a normal distribution (Fig 4) and we noted approximately 350 genes that were significantly up-regulated in the Jyg MC (B) cell line (Fig 5). We categorized the genes based on functions and found up-regulated gene expression profiles of interest, including astrocyte-derived cytokine receptors (Fig 6).

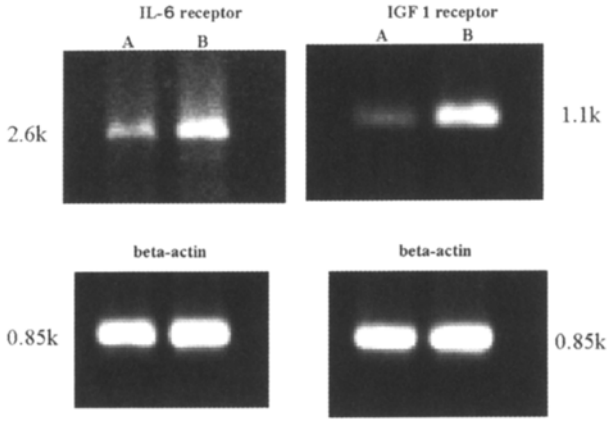

Fig 7. Results of RT-PCR. A, Jyg MC (A); B, Jyg MC (B). We used beta-actin as a control. We confirmed the correlation between the expression of astrocyte derived cytokine receptor genes and the formation of brain metastasis by breast cancer.

Detection of IL-6R and IGF-1R Status Complex in Jyg MC (A) and Jyg MC (B) Variants

We confirmed the correlation between the expression of astrocyte derived cytokine receptor genes and the tendencies of breast cancer to metastasize to brain using RT-PCR. Messenger RNA expression for IL-6R and IGF-1R was determined by RT-PCR analysis as described in materials and methods. The intensities corresponding to IL-6R and IGF-1R were expressed as ratios compared with beta-actin in each cell line and used to compare the differences among cell variants. We found that Jyg MC (B) cells had increased expression of certain genes compared with Jyg MC (A). These results were corroborated by cDNA microarray analysis as shown in Fig 7.

Discussion

The cDNA Microarray technique is very useful for the comprehensive analysis of gene expression profiles. In this study, we have shown that cDNA microarray analysis provided many candidate genes that may be associated with the establishment of brain metastasis from breast cancer. In addition, some of the possible genes were confirmed by accordant results agreed from RT-PCR.

In the central nervous system, glial cells, which have traditionally been viewed as providing structural support for neurons, have also been reported to play an important role in maintaining homeostasis, as evidenced by their potential to perform a variety of functions are usually attributed to accessory cells of the immune system, and the capacity of astrocytes to respond to and/or produce cytokines in response to extracellular stimuli has been studied widely. Astrocytes can synthesize IL-1, IL-3, IL-6, IFN-gamma, TNF-alpha, TGF-beta, IGF-1, and platelet-derived growth factor (PDGF), among many other cytokines. They can also express major histocompatibility complexes and serve as antigen-presenting cells and targets for immune responses. Moreover, the cytoplasm of astrocytes extends to endothelial cells and forms end-foot processes that surround central nervous system microvessels, forming the characteristically tight junctions of the blood-brain barrier.

It is suggested that astrocytes contribute to the specific microenvironment that selectively supports the growth of metastatic cells, once they have crossed the blood-brain barrier. We base this on our findings that astrocytes provide a good and specific cell adhesive substrate for breast cancer cell attachment, and also on the fact that they are a source of cytokines and growth factors, which may modulate metastatic cell growth. The induction of reactive astrocytes during tumor cell invasion and lodgment in the brain promotes the selection of trophic factors and growth factors from different glial cells, which aids in the invasion, survival, and growth of breast cancer cells in the central nervous system. Thus, the outcome of brain metastasis may depend on interactions with, and responsiveness to, astrocytes and astrocyte-releaced molecules. Among the different cytokines that are produced by astrocytes, IL-6 seems to play a specific role on brain metastatic cells. Indeed, IL-6 has been demonstrated to induce growth inhibition and enhanced motility in some breast cancer cells lines[12]. Although we did not detect increased motility in the breast cancer cell lines after IL-6 treatment[13], we found that brain metastatic cell sublines were refractory to exogenous IL-6-mediated growth inhibition. Thus, escape from the negative control exerted by this cytokine may represent a selective advantage that permits the growth of metastatic cell sublines in the brain.

Resistance to IL-6 growth inhibition has been found to correlate with advanced progression in melanoma[14] and with the estrogen receptor- negative phenotype in some breast cancer cell lines[15]. In contrast to other reports[16, 17] that implicate the lack of expression of IL-6R as cause of IL-6 resistance, we found that all breast cancer cell lines expressed both the binding and signal-transducing subunits of the IL-6R complex. Interestingly, IL-6R was slightly

increased in brain metastatic sublines compared with other cell variants, as demonstrated by Northern and Western blot analysis.

We considered the possible anticrime growth regulatory effects of this cytokine in our system because several studies have demonstrated that ER (−) breast cancer cell lines can secrete active IL-6[15]. Endogenous production of IL-6 was assessed by Nothern blot analysis, revealing similar mRNA levels for IL-6 in all breast cancer cell lines. Whatever the mechanism governing IL-6 expression, the question remains whether this cytokine has any function in anticrime and/or paracrine growth inhibition or stimulation of cell populations, as is the case in other tumors[18, 19].

In the present study, we confirmed these previous outcomes using microarray, and found that many kinds of genes, including extracted sequence tags (EST), were up-regulated in the highly brain metastatic cell line. These results may in turn dictate differences in organ specificity of metastatic cells that require or are sensitive to growth factors in the microenvironment.

However, we were unable to explain how many genes play a specific role on brain metastasis. Additionally, we are convinced that further detailed investigations with cDNA microarray will be worthwhile for determining which genes may regulate the mechanism of brain metastasis.

Acknowledgments

This study has been supported by Special Coordination Funds for promoting Science and Technology and a Research Grant for the RIKEN Genome Exploration Research Project from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, Special Coordination Funds for Promoting the Bioresource Program from RIKEN Tsukuba and CREST (Core Research for Evolutional Science and Technology) and ACT-JST (Research and Development for Applying Advanced Computational Science and Technology) of Japan Science and Technology Corporation (JST). This work was also supported by a Grant-in-Aid for Scientific Research on Priority Areas and Human Genome Program, from the Ministry of Education, Science and Culture, and by a Grant-in-Aid for a Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare to Y. H.

This study was supported in part by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of the Japanese Government to YO.

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