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Potential in a Single Cancer Cell to Produce Heterogeneous Morphology, Radiosensitivity and Gene Expression

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Dedifferentiation/Morphology/Radiosensitivity/Gene expression/Microarray.

Morphologically heterogeneous colonies were formed from a cultured cell line (KYSE70) established from one human esophageal carcinoma tissue. Two subclones were separated from a single clone (clone13) of KYSE70 cells. One subclone (clone13-3G) formed mainly mounding colonies and the other (clone 13-6G) formed flat, diffusive colonies. X-irradiation stimulated the cells to dedifferentiate from the mounding state to the flat, diffusive state. Clone 13-6G cells were more radiosensitive than the other 3 cell lines. Clustering analysis for gene expression level by oligonucleotide microarray demonstrated that in the radiosensitive clone13-6G cells, expression of genes involved in cell adhesion was upregulated, but genes involved in the response to DNA damage stimulus were downregulated. The data demonstrated that a single cancer cell had the potential to produce progeny heterogeneous in terms of morphology, radiation sensitivity and gene expression, and irradiation enhanced the dedifferentiation of cancer cells.

INTRODUCTION

It is well known that the cells in cancer tissue are heterogeneous in terms of morphology and differentiation status, even if the cancer tissue consists of progeny developed from a single neoplastic cell.[1)] Morphological changes in the cytoplasm and/or the nuclei are frequently observed in different areas of a biopsied cancer tissue after radiotherapy.[2,3)] The reasons why such morphologically different cells appeared among the exposed cell population have not yet been disclosed.[4)] Usually, the histological grades of tumor types at recurrence after radiotherapy shift to higher levels than those at initial diagnosis.[5,6)]

There are two conventional explanations for the appearance of histologically heterogeneous cells in a tumor tissue. Some researchers consider that radiation transforms the cancer cells so that they become radioresistant,[7–9)] others hypothesize that a large fraction of radiosensitive cells in the original tumor tissue is selectively killed and a small fraction of radioresistant cells actively grows and replaces the radiosensitive fraction.[10)]

Also, the cultured cancer cells have often been characterized by their morphological heterogeneity. But, the extent of cellular morphological heterogeneity can't be evaluated easily. When we assessed the dose-survival responses of 31 cultured human esophageal carcinoma cell lines[11,12)] by a colony-formation assay, we found that 1 cell line (KYSE70) formed morphologically variable colonies in one dish: a densely mounding type (M-type), a flat, diffusive type (F-type), and a type with mixed mounding and flat cells (M/F-type). Classification of colonies into three types made possible to evaluate the extent of morphological heterogeneity. The proportion of M-type colonies decreased with increasing X-ray dose. In contrast, the proportions of M/F- and F-type colonies increased with increasing dose. X-ray irradiation seemed to stimulate the cells to transform from M-type to F-type. The cell renewal or the clonogenic growth of cancer cell is a cell division in which the proliferating potential of progenies is similar to that of the parental cell. However, if a committed progenitor cell is produced, it may have a characteristic phenotype which must be different from the parental cell. The committed progenitor cell has an extensive ability to proliferate, destined to eventually become terminally differentiated and stop dividing. Therefore, the transformation process from M-type to F-type found in this study should not be expressed as 'differentiation', because this phenomenon was observed in the cancer cells and because the F-type colony cells never stop dividing. The term 'ded-

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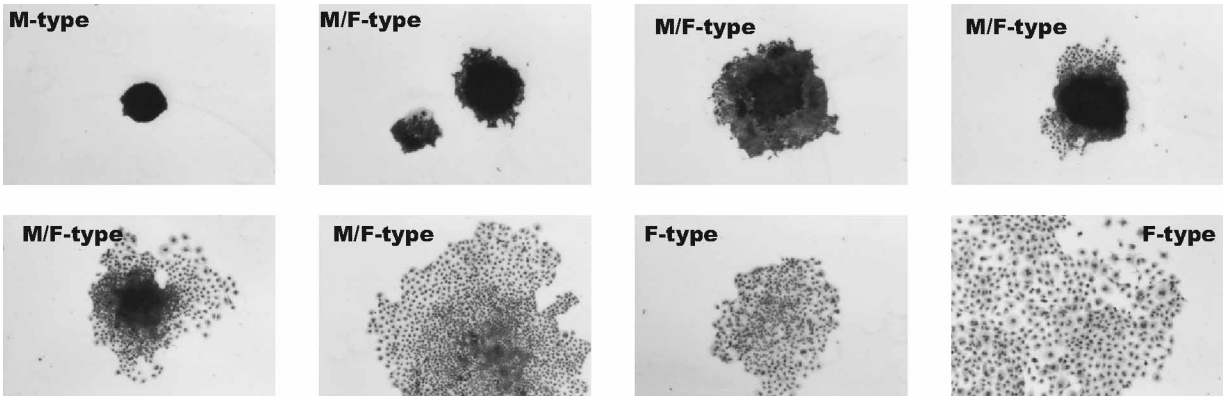

Fig. 1. Morphologically variable colonies observed in a 10-cm dish in which 200 cells of KYSE70 were inoculated. Colonies were stained with Giemsa. The colonies were classified into 3 types: the 'M-type' colony consisting of only mounding cells, the 'F-type' colony consisting of only flat, diffusive cells, and the 'M/F-type' colony consisting of mounding cells and flat, diffusive cells.

ifferentiation' has often been used in the clinical radiology study.[4–6] Here, we therefore refer to the KYSE70 cells' dedifferentiation-like morphological alteration as '*in vitro* dedifferentiation'. We isolated M- and F-type colony cells from a clone of the KYSE70 cells. The radiosensitivity of the isolated F-type colony cells was significantly higher than that of the original cells. Genes involved in tumor invasion, cell motility, and cell-shape change were upregulated in these F-type cells. Our data suggest that one tumor cell had the potential to produce heterogeneity in morphology, radiosensitivity, and gene expression, and that X irradiation enhanced the *in vitro* dedifferentiation. Further, our data provide a clue as to why histologically heterogeneous cells appear in the tissues of tumors that recur after radiotherapy.

MATERIALS AND METHODS

Cells and culture medium

The origins and other details of KYSE cell lines have been described elsewhere.[11,12] We established KYSE70 cells from poorly differentiated esophageal cancer tissue that had developed in a 77-year-old Japanese male.[12] Alpha MEM (Sigma, Tokyo, Japan) supplemented with L-glutamine and 10% fetal calf serum was used for the maintenance of all KYSE cell lines in all experiments. We used 0.25% trypsin plus 0.02% EDTA solution to harvest the cells.

Isolation of clones

We inoculated 200 μL of KYSE70 cell suspension (8 cells/mL) into each well of four 96-well Titertek plates, and incubated the cells in 5% CO_2 at 37°C for 2 weeks. Twelve M-type colonies were isolated. Because of its growth condition, clone 13 was selected for the next cloning. Then, 200 μL of clone13 cell suspension (8 cells/mL) was inoculated into each well of four plates of 96-well Titertek plates, and incubated in 5% CO_2 at 37°C for 2 weeks. Twelve M-type and 12 F-type colonies were isolated.

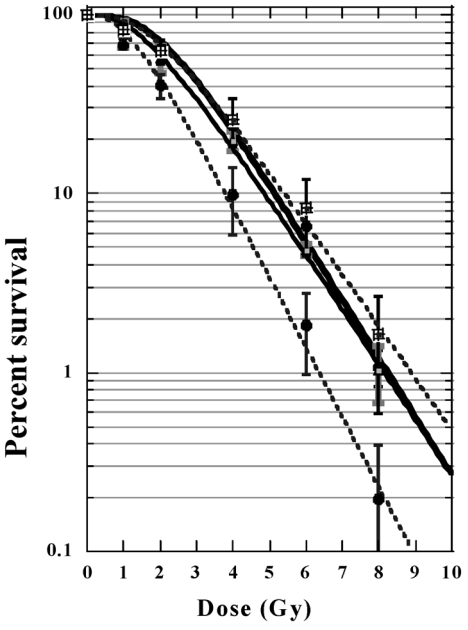

Fig. 2. X-ray dose-survival responses of KYSE70 (closed circles with bold line), clone13 (triangles with bold line), clone13-3G (squares with dotted line) and clone13-6G (closed circles with dotted line) cells. Bars indicate the mean ± S.D. of 3 independent experiments in the case of KYSE70 and clone13 cells or 6 independent experiments in the case of clone13-3G and clone13-6G cells, respectively. Clone 13-6G cells were more radiosensitive than the other 3 cell lines. Colony-forming ability (or plating efficiency) was 50–80% for each cell line.

Table 1. Values of survival parameters of KYSE70 cells and their derivatives (parameters are defined in the text.)

Cells	D_0 (Gy)	D_{10}(Gy)	D_q (Gy)	N
KYSE70	1.28	5.24	2.27	5.8
Clone13	1.35	4.95	1.82	3.8
Clone13-3G	1.44	5.5	2.17	4.5
Clone13-6G	1.02	4.09	1.74	5.5

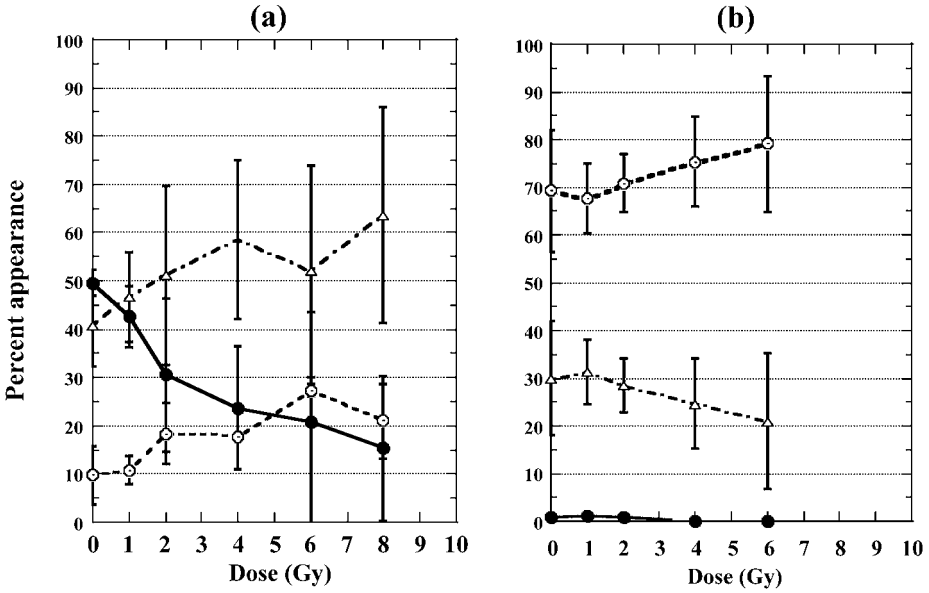

Fig. 3. Morphological changes in colonies after exposure to various X ray doses. Colonies produced in response to each dose (Fig. 2) for the clone13-3G (a) or the clone13-6G (b) were classified into M-type (●), M/F-type (△) and F-type(○). Bars indicate the mean ± S.D. of 6 independent experiments.

X-irradiation

Actively growing cells were harvested and suspended in growth medium, after which 5×10^4 cells (1 mL) were put into a plastic test tube and irradiated with X rays at room temperature. The X-ray generator (Shimadzu, Kyoto, Japan) was operated at 200 kVp and 20 mA, with 0.5 mm Cu plus 0.5 mm Al external filtration. The dose proximal to the cells was measured with a Victoreen condenser chamber (Victoreen Instruments, A-2340Moedling, Austria). The dose rate was 1.0 Gy/min. Cells were exposed to total doses of 0, 1, 2, 4, 6, or 8 Gy.

Clonogenic assay for cell survival

Immediately after irradiation, appropriate numbers of cells were seeded into plastic culture dishes and cultured for 2 weeks in 95% air plus 5% CO_2 at 37°C. The colonies were fixed with ethanol and stained with 2% Giemsa. Colonies composed of 50 or more cells were scored.

The dose responses were analyzed by using a multi-target model:

$$S = 100[1 - (1 - e^{-D/Do})^N]$$

where D is the dose in Gray (Gy) and S is the surviving fraction at dose D. D_0 (the dose that causes the straight-line portion of the survival curve to decrease to 37%) is 1/e. D_q, the dose at shoulder part of dose-survival curve, was also calculated. The intercept, N, of each survival curve was estimated by least-squares regression analysis as the parameter of nonlinear function.[13] Dose-survival curves were computer generated by the KaleidaGraph program (Synergy Software, Reading, PA. USA).

Microarray study

A custom-made oligonucleotide microarray (Agilent Technologies, Palo Alto, CA. USA) was used for these studies. Sequences for the 60-mer custom oligonucleotide microarray were selected from RefSeq. (Information was extracted in March 2002 from http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html). This array consisted of 22500 total spots, including 636 kinds of control spot and 20000 unique genes.

Total RNA was isolated with an RNeasy-Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The extracted total RNAs were further treated for 1h at 37°C with 10 units of RNase-free DNase I (TaKaRa, Kyoto, Japan) in the presence of 1 unit of RNase inhib-

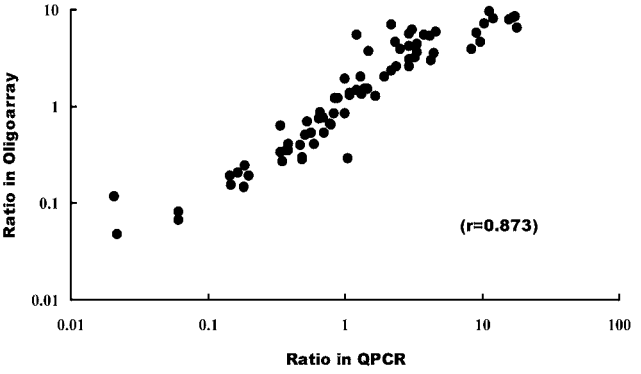

Fig. 4. Correlation of expression levels of 72 genes as determined by oligoarray and quantitative PCR (QPCR). 72 genes were randomly selected by their expression levels in the oligoarray. Correlation coefficient between two methods was 0.873.

itor (Toyobo, Osaka, Japan) and RNeasy spin columns (Qiagen) to remove any residual genomic DNA.
CyDye-labeled cRNA was generated by *in vitro* transcription with a Fluorescent Linear Amplification Kit (Agilent Technologies) in accordance with the manufacturer's instructions. For the direct comparison of expression patterns between clone13-3G and -6G cells, 1 μg Cy3-labeled cRNA from clone13-6G cells was mixed with an equal amount of Cy5-labeled cRNA from clone13-3G cells. For clustering analysis of KYSE70 and its 2 derivative cell lines (clone13-3G and clone13-6G), cRNA from each cell line was labeled with Cy3 and mixed with an equal amount of Cy5-labeled global reference RNA (mixture of human brain: cat#64020-1, human fetal brain: cat#64019-1, human fetal liver: cat#64018-1, human kidney: cat#64030-1, human lung: cat#64023-1, human placenta: cat#64024-1, human prostate: cat#64038-1, human small intestine: cat#64039-1, human testis: cat#64027-1, human thymus: cat#64028-1, human uterus: cat#64029-1, Biosciences Clontech, Tokyo). Hybridization was performed according to the *in situ* Hybridization Reagent Kit protocols (Agilent Technologies), scanned with a confocal laser scanner (Agilent Technologies) and quantified using Feature Extraction software (Agilent Technologies). Gene expression data were visualized and analyzed with Resolver software (Rosetta Inpharmatics, Seattle, WA, USA).

Real-time quantitative PCR (Q-PCR)

Reverse transcription was carried out at 37°C for 1hr with an Omniscript reverse transcriptase kit (Qiagen) in a 20-μL reaction mixture containing 2 μg of total RNA and 1 μM oligo $(dT)_{12–18}$ primer, 500 μM of each dNTP, 10units of RNase inhibitor, and 8 units of Omniscript reverse transcriptase. Q-PCR primer sequences for individual genes were selected with the aid of the Primer 3 program.[14] Q-PCR were performed with iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and with an iCycler iQ Real-Time PCR Detection System (Bio-Rad). The PCR program was initiated with 15min at 95°C for activating the HotStarTaq DNA polymerase, followed by 60 cycles, each of 20 s at 95°C for denaturing , 30 s at 62°C for annealing, and 30 sec at 72°C for extension.

RESULTS

Appearance of morphologically variable colonies in a dish

Morphologically variable colonies were observed in one 10-cm-diameter plastic dish in which 200 cells of KYSE70 had been inoculated (Fig. 1). The colonies were classified into 3 types: the 'M-type' colony consisting of only mounding cells, the 'F-type' colony consisting of only flat, diffusive cells, and the 'M/F-type' colony consisting of mounding cells and flat, diffusive cells.

X-ray dose-responses

It was possible that the KYSE70 cell population consisted of different types of cells that were already present in the original tumor. We therefore isolated several clones from the KYSE70 cells, of which clone13 (an M-type colony) was chosen for the next cell separation. Twelve M-type colonies and 12 F-type colonies were isolated from the clone 13 cells and maintained for more than half a year. Twenty-three of the 24 isolates formed various types of colonies from the M-type to the F-type. Ninety percent of clone13-3G cells formed M- and the M/F-type colonies. The radiation sensitivities of the parental KYSE70 cell line and its derivatives were determined by colony-formation assay (Fig. 2). Clone13-6G cells were more radiosensitive than the other 3 cell lines. The 3 dose-survival parameters (D_0, D_{10}, and D_q values) of clone 13-6G cells were smaller than those of other three cell lines (Table 1).

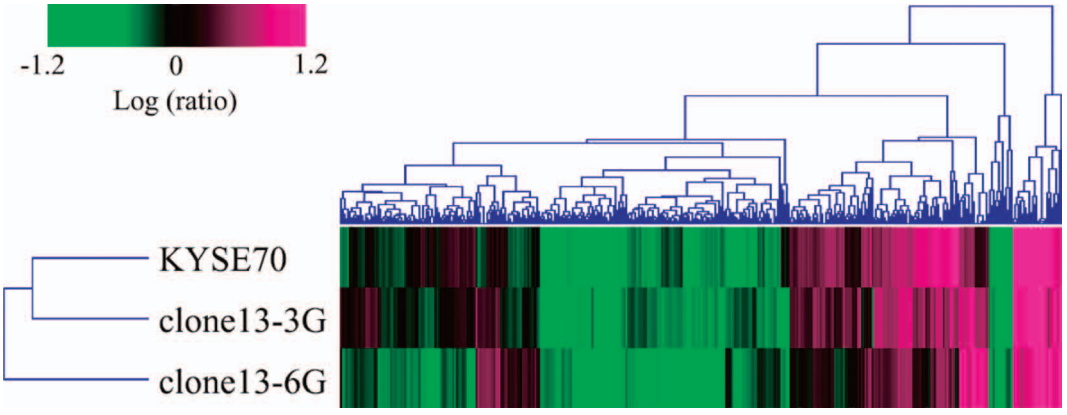

Fig. 5. Two-dimensional hierarchical clustering of genes potentially involved in *in vitro* dedifferentiation. Genes that showed 2-fold differences in expression level ($P \leqq 0.01$) between the clone13-3G and the clone13-6G cell lines were searched. 122 genes were upregulated in the clone13-3G cells and 283 genes, in the clone13-6G cells. Total 405 genes were cluster-analyzed. Clone13-6G cells were less closely related to the parental KYSE70 cells than were clone13-3G cells.

Table 2. Gene Ontology at level 4 for the Biological Process and the Molecular Function: classification of genes upregulated in clone13-3G or-6G cells. Fold change means the ratio in expression level between the 2 cell lines.

	Up-regulated genes in clone13-3G			Up-regulated genes in clone13-6G		
Biological and molecular function	Gene symbol	Accession No.	Fold change	Gene symbol	Accession No.	Fold change
Cell adhesion	CDH11	NM_001797	5.27	ARHE	NM_005168	2.37
				ARHGDIB	NM_001175	3.86
				COL5A1	XM_088423	2.47
				CYR61	NM_001554	2.93
				DLC1	MN_006094	8.36
				ESDN	NM_080927	2.94
				LAMC2	NM_005562	2.23
				NELL2	NM_006159	2.5
				OLR1	NM_002543	2.86
				PCDH22	NM_032971	2.1
				PTPNS1	NM_080792	2.64
				TGFB1	NM_000358	8.06
Cell death	CXCR4	NM_003467	2.56	CASP1	NM_001223	2.45
	P8	NM_012385	2.11	IL1B	NM_000576	2.97
	PDCD6	NM_013232	2.74	TNFAIP3	NM_006290	2.49
	PDCD8	NM_004208	2			
Cell signaling	SLC1A3	NM_004172	2.24	ADM	NM_001124	3
				BST2	NM_004335	2.21
				CCL26	NM_006072	4.31
				FURIN	NM_002569	2.23
				IL15	NM_000585	2.21
				IL1B	NM_000576	2.97
				INHBB	NM_002193	6.15
				SEMA3B	NM_004636	2.5
				WNT3	NM_030753	2.31
Regulation of CDK activity	BCCIP	NM_016567	2.32	CDKN1C	NM_000076	2.41
Regulation of cell proliferation	CXCL1	NM_001511	2.29	CDKN1C	NM_000076	2.41
				IL15	NM_000585	2.21
				IL1B	NM_000576	2.97
Response to DNA damage stimulus	CHES1	XM_007389	2.16			
	PDCD8	NM_004208	2			
Response to hypoxia				ARNT2	NM_014862	2.4
Caspase activator activity				CASP1	NM_001223	2.35
Cytoskeletal protein binding				CDC42EP3	XM_087188	2.05
				LCP1	NM_002298	3.1

Colonies observed in 12 independent experiments for X-ray dose survival on clone 13-3G and -6G cells (Fig. 2) were classified into 3 morphological types (Fig. 3). In clone13-3G, the proportion of M-type colonies decreased with increasing radiation dose, but those of M/F- and F-type colonies increased (Fig. 3a). Few M-type colonies were observed in dishes inoculated with cells of clone13-6G; with increasing radiation dose, the proportion of F-type colonies increased, but that of M/F-type colonies decreased (Fig. 3b). Because clone13-6G cells were more radiosensitive than clone13-3G cells (Fig. 2), we cannot say that X-ray-induced lethal effect was larger in M-type cells than in M/F- and F-type cells. We can hypothesize that radiation transformed some M-type cells into M/F- or the F-type cells and some M/F-type cells to the F-type cells. *In vitro* dedifferentiation was directed from M-type cells to F-type cells.

Microarray data and real-time RT-PCR analyses

We used the oligonucleotide microarray to compare the expression patterns of about 20000 genes in clone13-3G and -6G cells. Some genes were upregulated in these clones.

To validate the oligoarray data, we compared it with quantitative real-time polymerase chain reaction (QPCR) for 72 genes which were randomly selected from the oligoarray to give a variable range of expression levels (Fig. 4). A clear correlation was found in expression levels as determined by the 2 methods (correlation coefficient = 0.873).

To identify genes that were expressed at high levels in clone13-3G or clone13-6G cells, we conducted a direct comparison of gene expression levels by an oligonucleotide microarray on which a mixture of Cy-dye-labeled cRNA extracted from Clone13-3G and -6G cells had been hybridized, and we searched genes that showed 2-fold differences in expression level ($P \leqq 0.01$) between the 2 cell lines. A total of 122 genes were upregulated in the clone13-3G cells and 283 genes were upregulated in the clone13-6G cells.
These 405 genes were cluster-analyzed with an oligonucleotide microarray on which the mixture of dye-labeled global reference RNA and dye-labeled clone13-3G RNA or -6G RNA had been hybridized. Figure 5 demonstrates that clone13-6G cells were less closely related to the parental KYSE70 cells than were clone13-3G cells.

By Gene Ontology[15] it was possible to predict the functions of 227 of 405 genes; 60 were upregulated in clone13-3G and 167, in clone13-6G. Of these, we selected 39 by Gene Ontology at level 4 for Biological Process and 3, at level 4 for Molecular Function. In the categories of cell adhesion, cell-cell signaling, and cytoskeletal protein binding, twenty-three genes were upregulated in clone13-6G cells, which grew and diffused on the culture vessel, but only two genes (*CDH11* and *SLC1A3*), in clone13-3G cells. Genes (*CHES1* and *PDCD8*) involved in DNA damage and repair were downregulated in clone13-6G cells, which demonstrated high radiosensitivity.

DISCUSSION

Morphological heterogeneity is frequently observed in the cultured cells *in vitro*.[11,12,16] Usually, these alterations are induced by intracellular and/or micro-environmental alterations, and are considered as reversible. It is very difficult to classify cultured human cancer cells by their morphology. We found that one cultured human cancer cell line (KYSE70 cells) formed various types of colony in a culture dish. We classified the colonies into 3 groups according to their morphology (Fig. 1). The M/F-type colonies might not be formed by the fusion of M- or F-type colonies, because the *in vitro* dedifferentiation was directed from the M-type cells to the F-type cells (Fig. 3). The progeny of M-type cells may often be committed to F-type cells during clonal expansion. The appearance of F-type cells among the M-type siblings may therefore explain the formation of M/F-type colonies. It was difficult to maintain cells in the M- or F-type morphology. We isolated 12 M-type colonies and 12 F-type colonies, but 23 of the 24 isolates produced M-, M/F- and F-type colonies in the 1 dish (Data not shown). Only clone13-6G cells formed few M-type colonies (Fig. 2). The proportion of M/F- and F-type colonies compared with M-type colonies increased with increasing X-ray dose (Table 1 and Fig. 3). Because clone13-6G (F-type) cells were more radiosensitive than clone13-3G (M-type) cells (Fig. 3), M-type colony-forming cells might not be preferentially killed. Instead, X-irradiation stimulated the transition from M-type to F-type via M/F-type. The results also suggest that the *in vitro* dedifferentiation was directed from M-type to the F-type via M/F-type.

We used Gene Ontology[15] to predict the functions of 227 genes that were up- or downregulated more than 2-fold in clone13-3G cells or clone13-6G cells, each other (Table 2). It is very interesting that 12 genes (*ARHE*, *ARHGDIB*, *COL5A1*, *CYR61*, *DLC1*, *ESDN*, *LAMC2*, *NELL2*, *OLR1*, *PCDH11Y*, *PTPNS1* and *TGFB1*) involved in cell adhesion were upregulated in the clone13-6G cells. These genes may play a role in attachment to the culture vessel and mobility of clone 13-6G cells. Only 1 adhesion gene (*CDH11*) was upregulated in clone13-3G cells that did not form flat colonies.
The *LOXL2* gene was not selected by our Gene Ontology analysis at level 4, although its upregulation was 3.1-fold higher in clone13-6G cells than in clone13-3G cells.
Kirschmann *et al.* demonstrated that lysyl oxidase (*LOX*) expression was upregulated to a greater extent in a highly invasive/metastatic human breast cancer cell line (MDA-MB-231) than in a poorly invasive/nonmetastatic breast cell line (MCF-7),[17] and that modulation of *LOX* and *LOXL2* expression and function in breast cancer cells affects *in vitro* cellular invasive activity.[18] Akiri *et al.*[19] transfected full-length LOXL2 (or LOR-1) cDNA to MCF-7 cells. LOXL2-

expressing MCF-7 cells invaded the pseudocapsules surrounding the tumors, and these workers concluded that *LOXL2* promotes tumor invasiveness and fibrosis.

The Rho family of GTPases plays key roles in the regulation of cell motility and morphogenesis. Joberty *et al*.[20] demonstrated that Borg proteins (*Borg2* = *CDC42EP3* in the category of Cytoskeletal protein binding of Table 2) function as negative regulators of Rho GTPase signaling. Hirsch *et al*.[21] demonstrated that *CEPs* (*CEP3* = *CDC42EP3* in Table 2) act downstream of Cdc42 to induce actin filament assembly, leading to cell shape changes. *CDC42EP3* was also upregulated twice as much in clone13-6G cells as in clone13-3G cells. *LOXL2* and *CDC42EP3* may contribute to the diffusive expansion property of clone13-6G cell colonies.

Interestingly, two genes (*CHES1* and *PDCD8*) responsible to DNA damage stimulus were downregulated in the clone13-6G cells. *PDCD8* (= apoptosis-inducing factor: *AIF* in ref.24) is released from mitochondria and decrease oxidative stress.[22] Pati *et al*.[23] isolated *CHES1* and found that CHES1 suppressed a number of DNA damage-activated checkpoint mutations in *S. cerevisiae*. They demonstrated that *CHES1* confers increased survival after the exposure to UV, X rays and methylmethane sulfonate. Down-regulation of *CHES1* and *PDCD8* in clone13-6G cells might be correlated with the enhanced radiosensitivity. Further, many genes with slightly altered expression may be involved in this enhanced radiosensitivity.

Morphologically heterogeneous cells have been frequently observed not only in primary tumors,[1] but also in metastatic[24] or recurrent tumors[25] after radiation therapy, although it is still not known whether such morphological alterations are reversible or irreversible. Tumor recurrence is a serious problem for cancer patients, because the recurrent tumor is usually more malignant than the primary tumor.[4,5] It is well known that recurrent tumors are usually resistant to further therapeutic radiation.[26] Two hypotheses might explain this radioresitantance. The first is that the irradiated cells become committed to dedifferentiation and thus acquire radiotolerance. The second is that the large fraction of radiosensitive cells in the original tumor tissue is selectively killed by the repeat radiation, and the small fraction of radioresistant cells then actively grows and predominates. Our *in vitro* data demonstrated the possibility that dedifferentiated cells were more radiosensitive than were undifferentiated cells. Therefore, our data may not square with either of above 2 hypotheses. Whether the dedifferentiated cells are radiosensitive or -resistant may not be the major problem. The serious problem may be the induction of morphologically diffusive cells and their clonal expansion in the cancer tissue. Our data suggest a third hypothesis that the diffusively growing potential can be induced in the non-diffusively growing cancer cells by the X irradiation. If these diffusive cells are more active in migrating into other tissues than the mounding cells, then this would explain why tumors consisting of diffusive cells are more malignant and/or metastatic than tumors consisting of non-diffusive cells.

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