Microarray Analysis of Temporal Gene Responses to Ionizing Radiation in Two Glioblastoma Cell Lines: Up-regulation of DNA Repair Genes

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Radiosensitivity/DNA repair/Glioblastoma/DNA microarray.

To determine the patterns of gene expression responsible for the radiosensitivity of glioblastoma cells, we analyzed transcriptional changes after ionizing radiation in different cell lines. After completing clonogenic survival assays, we selected two glioblastoma cell lines with different radiosensitivities. Subsequently, they were investigated by using the technique of DNA microarray, and we then categorized the upregulated genes into 10 groups. Between the two cell lines, the difference in the percentage of DNA repair/replication category was the largest, and this category was present at a greater percentage with radioresistant cell line U87MG. Moreover, among the commonly upregulated genes, the DNA repair/replication category was present in the largest percentage. These genes included G22P1 (Ku70) and XRCC5 (Ku80) genes known as important members of the nonhomologous end-joining (NHEJ) pathway of DNA double strand break (DSB) repair. Furthermore, cell line that specifically upregulated genes included the members of major pathways of DNA DSB or single strand damage repair. These pathways were not only NHEJ, but also homologous recombination (HR) and postreplication repair (PRR). In conclusion, the distribution of genes involved in the DNA repair/replication category was most different between two human glioblastoma cell lines of different radiosensitivities. Among commonly upregulated genes, the DNA repair/replication category was present in the largest percentage.

INTRODUCTION

Glioblastoma is the most malignant astrocytic tumor, and radiotherapy is an important component of multimodal treatment. Although radiotherapy prolonged the survival of patients with this disease,1) the recurrence rate is still very high. Biological modifications during radiotherapy based on genetic information may improve the efficacy of radiotherapy for glioblastoma.2)

Many studies have been reported about correlations between gene expression and radiotherapeutic response,3–5) survival time after regrowth,4) and in vitro radiosensitivity.6–9) Furthermore, Rhee et al.10) reported evidence that cell lines of the same pathological origin could be highly heterogeneous in gene expression. Recent technological advances in DNA microarray have made it possible to profile the gene expression responses to ionizing radiation (IR) simultaneously. A variety of genes, including immediate-early and acute phase genes, DNA repair genes, cytokine, and growth factor genes have been proposed as members of the mechanisms by which cells survive after IR.11–13) Although these gene expression levels change quickly, a few studies reported time-course-related gene induction after IR. Here we report time-related changes and cell line specific patterns of gene expression after IR by using two glioblastoma cell lines of different radiosensitivities. Moreover, our methods can provide valuable information for designing effective radiotherapy of various tumor types.

MATERIALS AND METHODS

Cell lines, irradiation, and clonogenic assays

Human glioblastoma cell lines, U87MG, A172, and U138MG were obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium with 10% fetal calf serum and antibiotics (90 U penicillin/ml, 90 µg streptomycin/ml) at 37°C in an atmosphere of 5% CO2 in air. We used a 150 kVp X-ray machine (MBR-1505R2, Hitachi Medical Corporation, Japan) for irradiation with a 0.5 mm aluminum filter at a dose rate of 2 Gy/min. We measured the radiosensitivities of these cell lines with a standard clonogenic assay as described previously.14)
Direct DNA sequence of exon 7 of the TP53 gene in cell line A172

The total RNA was isolated from unirradiated A172 cells by the acid guanidinium thiocyanate-phenol-chloroform method. The first-strand complimentary DNA (cDNA) that served as the polymerase chain reaction (PCR) template was synthesized from total RNA (2 μg) by using reverse transcriptase (Super Script II, Life Technologies, Invitrogen Corp., Carlsbad, CA, USA) and oligo-dT primers. The following procedure was used for direct sequencing of PCR-amplified cDNA molecules: A 200-base pairs fragment spanning exon 7 of the TP53 gene was PCR-amplified from 0.5 μl of cDNA by using 0.4 μmol of each of the following primers: sense (5′-GTGTGGATTTGGATGACAG-3′) and antisense primers (5′-CACAAAACATGCACTCTGAAAACGC-3′), 1.6 μl dNTPs, 1.5 mmol MgCl₂, and 0.2 μl of Taq DNA polymerase (LA Taq, Takara Bio Inc., Japan). Subsequent procedures were performed according to Kyritsis et al.

Preparation of cells, fluorescent cDNA, and hybridization for the DNA microarray

We used U87MG and A172 cells for the analysis with the DNA microarray. Cells were grown to confluence and were irradiated with or without a single dose of 6 Gy as described above. Samples were collected at 3 times (i.e., 0.5, 1, and 6 h after irradiation). As a control, unirradiated cells were used and collected at the same times. Total RNAs were extracted from cells as described above. A fluorescent probe was synthesized by incorporating Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech) with an RNA Fluorescence Labeling Core Kit (Takara Bio Inc., Japan), using 25 μg of the total RNA described above and 25 U AMV reverse transcriptase (Takara Bio Inc., Japan). We labeled total RNA from irradiated cells with Cy3 and total RNA from unirradiated cells with Cy5 dyes. After purification, Cy3 and Cy5 probes were mixed with human Cot aT DNA, poly(dA), and yeast tRNA (5 × Competitor aT, Takara Bio Inc., Japan), then followed by ethanol precipitation. The mixture was resuspended in 15 μl of a hybridization solution (6 × SSC, 0.2% SDS, 5× Denhardt’s solution, 0.1 mg/ml salmon sperm DNA). Subsequently, we performed prehybridization, hybridization and washing according to the method of Taniguchi et al.

Scanning and analysis of DNA microarrays

We performed microarray analysis by using human Cancer CHIP Version 3.0 (Takara Bio Inc., Japan), in which 638 human cancer-related genes and housekeeping genes (listed on the home page of Takara Bio Inc., http://bio.takara.co.jp/BIO_EN/DNACHip_Download_e.htm) were spotted on a glass plate. Before each use of the Human Cancer CHIP Version 3.0, we performed microarray analysis as described above by using the TestARRAY Version 3.0 DNA chip (Takara Bio Inc., Japan) to check for labeling probes. Fluorescence intensities were captured with a laser confocal scanner; GMS 418 Array Scanner (Affymetrix). Subsequently, obtained data were analyzed with software: ImaGene version 4.1 (BioDiscovery Inc). For quality control, automatically marked spots that were not distinguishable from the background were removed. Negative values resulting from a subtraction of the background were removed. Eight signal spots of beta-actin cDNAs were used for normalization. After normalization, 2.7-fold differences in upregulated expression were used to identify the unchanged or altered genes. We arbitrarily chose this ratio of 2.7 as the cutoff value for matches of independent experiments and comparison of A172 and U87MG cells data, considering the manufacturer’s instruction that showed a twofold induction was significant. We used histograms for gene selection and confirmed these signals in scatter plots created by ImaGene software. The selected genes were checked by using the web site of UniGene (http://www.ncbi.nlm.nih.gov/UniGene/index.html). Annotations and functional classification of genes were based on PubMed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov/entrez/), LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/), GENE ONTOLOGYTM CONSORTIUM (http://www.geneontology.org/), Proteome database (http://www.proteome.com/), and other database web sites.

RESULTS

Radiosensitivities of cell lines

Figure 1 shows the radiation survival curves derived from colony formation assays of 3 cell lines. The radiosensitivity of A172 cells was the highest among 3 cell lines. On the other hand, the radiosensitivity of U87MG cells was the lowest. So we selected the U87MG and A172 cell lines for the analysis
because of their difference in radiosensitivity. The survival fraction of U87MG cells was slightly higher than for A172 cells at the dose of 3 Gy. In contrast, more apparent differences in the survival fraction were observed among the 3 cell lines at doses of 6 and 9 Gy. Taking into account these dose-dependent differences in the survival fraction and considering clinically relevant doses, we decided on 6 Gy at the irradiation dose for microarray analysis.

**Confirmed sequence of exon 7 of the TP53 gene in cell line A172**

In this study, we used 3 cell lines in the clonogenic assay because of their known TP53 gene status, and they were reported as wild-types.8,18,19) On the other hand, in previous studies mutations of the TP53 gene at exon 7 in the A172 cell line were also reported20). Therefore we used direct sequencing to examine a part of the coding region of the TP53 gene for mutations in the region, including exon 7 of the gene of cell line A172. No mutation was found in the region, and the TP53 gene of cell line A172 was used as a wild type.

**Analyses of gene expression after X-ray irradiation**

Table 1 shows the number of upregulated genes in the two cell lines with respect to time after irradiation. We used histograms for gene selection and confirmed these signals in scatter plots created by ImagenGene software (Fig. 2) as described in “MATERIALS AND METHODS.” To detect the immediate early and acute phase transcriptional responses, we set time points at 0.5, 1, and 6 h as described in “MATERIALS AND METHODS.”

**DISCUSSION**

This is the first study describing time course-dependent and cell-line specific patterns of gene expression after IR, using two human glioblastoma cell lines of different radiosensitivities. These cell lines were selected because of their known TP53 status as wild types and their radiosensitivities as a consequence of clonogenic assays. Time-course-dependent
Table 1. Upregulated genes.

| Category | Gene name                                                                 | Symbol |
|----------|---------------------------------------------------------------------------|--------|
|          | Induced time after irradiation (h)                                        |        |
|          | In A172                                                                   | In U87MG|
|          | 0.5 1 6                                                                  | 0.5 1 6 |
|          | **Apoptosis**                                                             |        |
|          | defender against cell death 1                                            | DAD1   |
|          | programmed cell death 10                                                 | PDCD10 |
|          | baculoviral IAP repeat-containing 3                                       | BIRC3  |
|          | BCL2/adenovirus E1B 19 kDa-interacting protein 3                          | BNIP3  |
|          | BCL2-related protein A1                                                   | BCL2A1 |
|          | caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase) | CASP1  |
|          | CD27-binding (Siva) protein                                               | SIVA   |
|          | death-associated protein                                                  | DAP    |
|          | DNA fragmentation factor, 45 kDa, alpha polypeptide                        | DFFA   |
|          | quinone oxidoreductase homolog                                            | PIG3   |
|          | p53-induced protein                                                       | PIG11  |
|          | v-rel avian reticuloendotheliosis viral oncogene homolog A (nuclear factor of kappa light polypeptide gene enhancer in B cells 3 [p65]) | RELA |
|          | growth arrest and DNA-damage-inducible 34                                | PPP1R15A |
|          | nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha | NFKBIA |
|          | Ras-related GTP-binding protein                                           | RAGA   |
|          | cytochrome c                                                              | HCS    |
|          | **Cell Communication/Signaling**                                           |        |
|          | integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)      | ITGA3  |
|          | interleukin 8                                                             | IL8    |
|          | epidermal growth factor receptor pathway substrate 8                      | EPS8   |
|          | active BCR-related gene                                                   | ABR    |
|          | EphB4                                                                     | EPHB4  |
|          | glia maturation factor, beta                                              | GMFB   |
|          | guanylate binding protein 1, interferon-inducible, 67 kDa                 | GBP1   |
|          | interferon-related developmental regulator 1                             | IFRD1  |
|          | signal sequence receptor, alpha (translocase-associated protein alpha)    | SSR1   |
|          | vascular endothelial growth factor C                                      | VEGFC  |
|          | fibronectin 1                                                             | FN1    |
|          | Rho GTPase activating protein 1                                            | ARHGAP |
|          | CD44 antigen (homing function and Indian blood group system)              | CD44   |
|          | guanine nucleotide binding protein (G protein), alpha-inhibiting activity polypeptide 3 | GNA13 |
|          | integrin beta 3-binding protein (beta 3-endonexin)                        | ITGB3BP|
|          | chondroitin sulfate proteoglycan 2 (versican)                             | CSPG2  |
|          | cadherin 13, H-cadherin (heart)                                           | CDH13  |
|          | paxillin                                                                  | PXN    |
|          | tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide | YWHAZ |
|          | **Cell Cycle**                                                            |        |
|          | CDC37 (cell division cycle 37, S. cerevisiae, homolog)                   | CDC37  |
|          | D123 gene product                                                         | D123   |
|          | nucleolar phosphoprotein p130                                             | NOLC1  |
|          | ras homolog gene family, member G (rho G)                                 | ARHG   |
|          | G1 to S phase transition 1                                                | GSPT1  |
|          | RAP1A, member of RAS oncogene family                                      | RAP1A  |
|          | CDC16 (cell division cycle 16, S. cerevisiae, homolog)                   | CDC16  |
|          | v-jun avian sarcoma virus 17 oncogene homolog                             | JUN    |
|          | CDC28 protein kinase 1                                                    | CKS1   |
|          | ras homolog gene family, member A                                         | ARHA   |
|          | **Cell Structure**                                                       |        |
|          | laminin, alpha 4                                                          | LAMA4  |

J. Radiat. Res., Vol. 45, No. 1 (2004); http://jrr.jstage.jst.go.jp
Table 1. (continued) Upregulated genes.

| Category                        | Gene name                        | Symbol | In A172 | In U87MG |
|---------------------------------|----------------------------------|--------|---------|---------|
| DNA Repair/Replication          |                                  |        |         |         |
| purine-rich element binding protein A | PURA                           |        | 1       |         |
| ataxia telangiectasia and Rad3 related | ATR                           |        | 1       |         |
| general transcription factor III, polypeptide 2 (44 kD subunit) | GTF2H2                     |        | 1       |         |
| proliferating cell nuclear antigen | PCNA                          |        | 1       |         |
| RAD52 (S. cerevisiae) homolog   | RAD52                           |        | 1       |         |
| adenine phosphoribosyltransferase | APRT                          |        | 1       |         |
| glutaredoxin (thioltransferase) | GLRX                           |        | 1       |         |
| centromere protein F (350/400 kD, mitosin) | CENPF                        |        | 1       |         |
| topoisomerase (DNA) II alpha (170 kD) | TOP2A                        |        | 1       |         |
| ubiquitin conjugating enzyme E2A (RAD6 homolog) | UBE2A                     |        | 1       |         |
| thyroid autoantigen 70 kD (Ku antigen) | G22P1                        | 1      | 1       |         |
| X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80 kD) | XRCC5                 | 1      | 1       | 1       | 1       |
| guanine monophosphate synthetase | GMP5                           | 1      | 1       |         |
| ribonucleotide reductase M1 polypeptide | RRMI                        | 1      | 1       |         |
| Metabolism/Energy               |                                  |        |         |         |
| phorbol-12-myristate-13-acetate-induced protein 1 | PMAIP1                  | 1      |         |         |
| aldolase A, fructose-bisphosphate | ALDOA                        | 1      | 1       |         |
| dihydrolipoyl dehydrogenase     | DPDY                           | 1      |         |         |
| hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome) | HPRT1                     | 1      |         |         |
| phosphoinositide-3-kinase, class 3 | PIK3C3                       | 1      |         |         |
| galactosidase, beta 1           | GLB1                           | 1      |         |         |
| glutathione peroxidase 1        | GPX1                           | 1      |         |         |
| glyceraldehyde-3-phosphate dehydrogenase | GAPD                      | 1      |         |         |
| phosphoglycerate kinase 1       | PGK1                           | 1      |         |         |
| lysophospholipase like          | MGLL                           | 1      |         |         |
| ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1 | ATP5F1                | 1      |         |         |
| malate dehydrogenase 1, NAD (soluble) | MDH1                        | 1      | 1       | 1       | 1       |
| ornithine decarboxylase 1       | ODC1                           | 1      | 1       |         |
| Protein Synthesis/Modification  |                                  |        |         |         |
| matrix metalloproteinase 3 (stromelysin 1, progelatinase) | MMP3                       | 1      | 1       |         |
| dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4 | DYRK4                    | 1      |         |         |
| F-box only protein 9            | FBXO9                          | 1      |         |         |
| superoxide dismutase 3, extracellular | SOD3                       | 1      |         |         |
| tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) | TIMP3                    | 1      |         |         |
| cathepsin L                     | CTS1                           | 1      |         |         |
| hsp70-interacting protein       | HSPBP1                         | 1      |         |         |

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Changes of upregulated genes were observed, and the functional classification of the upregulated genes showed interesting distribution patterns. The difference in the percentages of genes categorized in DNA repair/replication between the two cell lines was the greatest (Fig. 3). Differences in other functional gene groups were not so significant. Furthermore, in commonly upregulated genes, the DNA repair/replication category was present in the largest percentage. Therefore, there is a possibility that genes categorized as DNA repair/replication play a role in determining radiosensitivity in glioblastoma cells. The significance of this observation remains to be determined and may affect the design of future studies.

X-ray irradiation induces complex cellular responses.6,7 The complex molecular responses to genotoxic stress are medi-
ated by a variety of regulatory pathways. The transcription factor p53 plays a role in the cellular response to DNA-damage agents such as IR, but other pathways also play important roles. To simplify and to more easily compare the data of DNA microarray, we used cell lines carrying wild-type TP53. Many genes related to the DNA repair/replication were upregulated in the radioresistant U87MG cells in this study. DNA is the critical target of IR and unrepaired double-strand breaks (DSBs) are the lesions most responsible for IR-induced cell death. The ability of cells to repair DNA DSBs usually correlates with their radiosensitivity.

Some of the upregulated genes in this study play roles in DNA repair after IR. Ku70 and Ku80 are two subunits of Ku protein, which are central to nonhomologous end-joining (NHEJ). In the present study, Ku70 (G22P1) and Ku80 (XRCC5), genes were induced in both cell lines at the same time points. The ATR protein is physically recruited to the site of DNA damage in vivo, and is related to NHEJ. Recent findings reveal links between ATM- and ATR-dependent systems of DNA damage signaling and the activation of the homologous recombination (HR) pathway. RAD52 gene products play important roles in HR, and the defect of this gene leads to increased sensitivity to IR. RAD52 was also determined as one member in the DNA postreplication repair (PRR) pathway that is known to convert DNA damage-induced single stranded gaps into large molecular weight DNAs without actually removing the replication-blocking lesions.

UBE2A is a homolog of the RAD6 gene. RAD6 controls DNA PRR centrally, and RAD6 defines one of three radiation repair epistasis groups, to which belongs. Further analyses showed that both RAD6 and RAD18 mutants were sensitive to a wide range of DNA damaging agents and were defective in PRR activity.

As described above, some of the upregulated genes involved in DNA repair/replication appear to play important roles in major pathways of DNA double-strand break or single strand damage repair. Recent reports showed that gene expression and protein levels have a close correlation with radiosensitivity.

Some studies suggested that apoptotic responses are important factors determining radiosensitivity. However, the present study showed little difference in the gene distribution related to apoptosis between the two cell lines (Fig. 3) and no induction in commonly upregulated genes (Table 1). Rapid apoptotic responses after IR usually occur in only a limited number of cell types, such as those of myeloid and lymphoid lineages. We used glioblastoma cell lines, which are relative radioresistant among various tumor cells. U87MG and A172 cells used in the present study may be resistant to radiation-induced apoptosis, and therefore the difference in the distribution of the genes between these 2 cell lines may be slight.

In conclusion, the distribution of genes involved in the DNA repair/replication category was most different between two human glioblastoma cell lines of different radiosensitivities. Among commonly upregulated genes, the DNA repair/replication category was present in the largest percentage.

ACKNOWLEDGEMENTS

This study was in part supported by the High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by a Grant-in-Aid for Scientific Research (C), Japan Society for the Promotion of Science (14570878). We thank K. Ishido for instruction on DNA microarrays. We also thank I. Ogino and S. Kurihata for technical assistance.

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Received on March 23, 2003
1st Revision on July 7, 2003
2nd Revision on October 23, 2003
Accepted on October 27, 2003

J. Radiat. Res., Vol. 45, No. 1 (2004); http://jrr.jstage.jst.go.jp