Orthotopic Growth of Human Glioma Cells Quantitatively and Qualitatively Influences Radiation-Induced Changes in Gene Expression

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Abstract

The effect of radiation on gene expression has been most frequently studied using tissue culture models. To determine the influence of experimental growth condition on radiation-induced changes in gene expression, microarray analysis was done on two human glioma cell lines (U87 and U251) grown in tissue culture and as s.c. or i.c. xenografts. Compared with tissue culture, the number of genes, whose expression was affected by radiation in both cell lines, was increased in the s.c. xenografts and further increased in the orthotopic tumors. Furthermore, in each growth condition, radiation modulated the expression of a different set of genes. In addition, there were few commonly affected genes after irradiation of U87 and U251 in tissue culture, there were 729 common changes after orthotopic irradiation. These results indicate that the influence of the orthotopic environment on radiation-induced modulation of gene expression in glioma cells was both quantitative and qualitative. Moreover, they suggest that investigations of the functional consequence of radiation-induced gene expression will require accounting for experimental growth conditions. (Cancer Res 2005; 65(22): 10389-93)

Introduction

Defining the molecular determinants of tumor cell radiosensitivity is not only a long-standing goal of fundamental radiobiology but is a prerequisite for identifying potential targets for radiosensitizers. Accordingly, the use of microarray analysis as a genome-wide approach for describing radiation-induced modifications in gene expression has generated considerable interest. Such studies have typically employed cell lines grown and maintained as monolayer cultures to identify the gene expression profiles of irradiated cells (1–3). A critical assumption in the use of monolayer cultures, however, is that the molecules that regulate the radioresponse of tumor cells in situ simulate the complex signaling processes that mediate radioresponse in vitro. Thus, defining the gene expression profiles of irradiated tumor cells grown under in vitro growth conditions may generate a more clinically relevant understanding of cancer cell radiobiology. Moreover, rather than the commonly used s.c. xenografts, it would seem that additional insight could be obtained by analyzing tumor cells grown under orthotopic conditions. Towards this end, we have focused on human glioma cell lines. Of solid tumors, gliomas have been and continue to be among the most resistant to therapy. However, when human glioma cell lines are evaluated in monolayer culture, their radiosensitivities are not significantly different from cell lines originating from other histologies that typically respond to treatment (4). A possible explanation for this discrepancy is that the molecules that regulate glioma cell radioresponse in monolayer culture are not the same as those for glioma cells in situ. We have recently shown that the orthotopic growth has a profound effect on the basal gene expression profile of human glioma cells (5). Therefore, as an initial step in evaluating the contribution of the growth environment to the radioresponse of gliomas, we have used microarray analysis to determine the radiation-induced changes in gene expression for two human glioma cell lines grown as in vitro monolayer cultures, as s.c. leg tumor xenografts, and as i.c. xenograft tumors.

Materials and Methods

Cell lines and in vivo tumor model. The human glioma cell lines U251 and U87 (American Type Culture Collection, Gaithersburg, MD) were grown in RPMI 1640 containing glutamate (5 mmol/L) and 5% fetal bovine serum at 37°C in 5% CO2 and 95% room air. Tumor cells (5 × 106 cells) were injected s.c. into the right hind leg or i.c. as described previously (6). The s.c. tumors were harvested at 500 mm3 and i.c. tumors at 13.5 mm3, at which time the animals were symptomatic. Tumors were irradiated (6 Gy) using a Pantak (Solon, OH) X-ray source at a dose rate of 1.55 Gy/min with animals restrained in a custom jig.

RNA sample preparation, probe labeling, and microarray procedure. The microarray methods followed closely those of previous studies (5, 7). Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), passed through an RNeasy spin column, and amplified using RiboAmp RNA Kits (Arcturus, Mountain View, CA; ref. 7). Amplified RNA (1.5–3.0 μg) was reverse transcribed and labeled with Cy5-dUTP (experimental RNA) or Cy3-dUTP (Stratagene Universal Reference, La Jolla, CA). Each cDNA microarray chip contained 7,680 human cDNA clones (Rosetta 8K Human Array); methods for microarray hybridization and washing were described previously (8). Hybridized arrays were scanned with 10-μm resolution on a GenePix 4000A scanner (Axon Instruments, Inc., Foster City, CA). TIFF images were analyzed by GenePix Pro 4.0 software (Axon Instruments), and ratio intensity was determined and normalized with the center of ratio distribution of 1.0. All samples had a biological replicate, and each replicate was run on duplicate slides.

Data analysis. Raw intensity profiles were analyzed using the mAd tools (National Center for Biotechnology Information, NIH; ref. 7). Only spots with signal-to-background ratios of >2, a minimum background corrected signal of 250 counts, and 60% of pixels in the spots with an
intensity greater than a SD plus background were used. Scatter plots were generated and genes that differed by \( z \) 2-fold between the irradiated and nonirradiated samples were analyzed for functional significance using the program GOstat.\(^5\) This program obtains the Gene Ontology annotations from a database and generates a statistical analysis of the functional annotations that are overrepresented in the inputted list of genes (9), with a Benjamini correction for multiple comparisons included (10). The false discovery rate was determined using OC plus running in R (11).

### Results and Discussion

To determine whether the radiation-induced modulation of gene expression is affected by growth conditions, U87 cells were grown as in vitro monolayer cultures and as xenografts at two sites in nude mice: s.c. in the hind leg and i.c. The s.c. group was intended to simulate a standard experimental model for evaluating in vivo therapeutic response, and the i.c. group represents an orthotopic model. Cultures or xenografts were irradiated (6 Gy); 6 hours later, the irradiated and a corresponding untreated sample were collected and subjected to microarray analysis. For each growth condition, the gene expression profiles generated for irradiated and untreated U87 cells were compared using scatter plots with correlation coefficients used as an indicator of similarity (Fig. 1). Radiation induced relatively few changes in gene expression in cells grown in vitro \((r = 0.92)\). For cells grown as s.c. xenografts, there was a greater discordance between the gene expression profiles generated for irradiated and untreated samples \((r = 0.79)\). This increased susceptibility to radiation-induced changes in gene expression in s.c. U87 xenografts compared with tissue culture is consistent with the previous report by Khodarev et al. (2). However, as shown in Fig. 1C, an even greater change in gene expression induced by radiation was obtained from i.c. xenografts \((r = 0.60)\). The actual number of genes affected after irradiation of U87 cells under in vitro, s.c., and i.c. growth conditions was 219, 1,139, and 1,748, respectively (Fig. 1D).

In addition to an overall increase in the susceptibility of U87 cells grown in vivo to radiation-induced changes in gene expression, there were also considerable differences in the specific genes...
affected (Fig. 1D). Of the 219 genes whose expression was modified after in vitro irradiation, only 49 and 58 were also modified after irradiation of s.c. and i.c. xenografts, respectively, and only 24 of the gene expression changes were detected in each of the three growth conditions. Thus, the increase in the number of genes affected in the s.c. and i.c. models was not simply in addition to those affected under in vitro conditions. These data indicate that for each growth condition, radiation modulated the expression of a different set of genes.

To determine whether the influence of growth conditions on radiation-induced changes in gene expression was unique to U87 cells, the same study was done using the U251 cell line, which is also a frequently employed human glioma model. Gene expression profiles for irradiated and untreated cells were compared via scatter plot for the three growth conditions: in vitro cultures, s.c. xenografts, and i.c. xenografts (Fig. 2A-C). In contrast to U87 cells, the correlation coefficients comparing irradiated to untreated U251 cells were essentially the same for in vitro and s.c. conditions ($r = 0.93$ versus 0.92, respectively). However, as for U87 cells, irradiation of U251 cells grown i.c. resulted in considerably more changes in the gene expression profile ($r = 0.59$). The actual number of radiation-modulated genes in U251 cells was 83, 114, and 1,639 for in vitro, s.c., and i.c. growth conditions, respectively (Fig. 2D). Whereas the number of genes affected in vitro and s.c. were not as dramatically different as in the U87 cell model, there was clearly a different set of genes affected with only nine genes in common between the in vitro and s.c. conditions. The genes affected after irradiation of i.c. U251 cells also had little in common with those affected under the other growth conditions: only 34 and 28 genes affected in the i.c. model were also affected by radiation in the in vitro and s.c. models, respectively. There were no common genes affected by radiation in each of the three U251 model systems. Thus, as for U87 cells, radiation modulated the expression of different sets of genes in each of the three model systems.

Data generated from these two glioma cell lines suggest that compared with the two most frequently used experimental model systems (tissue culture and s.c. xenografts), cells grown under orthotopic i.c. conditions are more susceptible to radiation-induced changes in gene expression. Moreover, of the genes whose expression was modified by radiation in the in vitro and s.c. models, less than half were also modified in the i.c. xenografts. We conclude that the influence of i.c. environment on radiation-induced modulation of gene expression in glioma cells was not only quantitative but qualitative.

A consistent finding in the microarray-based analysis of radiation-induced changes in gene expression has been the lack of similarity or overlap between cell lines regarding the specific genes affected. This cell type specificity has been reported for tumor cell lines irradiated as in vitro cultures (1–3) and as s.c. xenografts (2) and has been attributed to the genetic background being the critical determinant of the gene expression changes induced by ionizing radiation (1, 3). However, as shown above, the normal brain environment has a profound effect on radiation-induced changes in gene expression. Moreover, we have recently reported that the gene expression profiles for untreated U87 and U251 glioma cells, whereas disparate in vitro, become less different as s.c. xenografts and actually similar when evaluated as orthotopic i.c. xenografts (5). To determine whether a similar situation exists for radiation-induced changes in gene expression, U87 and U251 expression profiles were compared after irradiation of cells grown in vitro and as s.c. and i.c. xenografts (Fig. 3). Comparison of the genes affected after irradiation of U87 and U251 cells grown in vitro revealed one commonly affected gene, consistent with previous reports (2). In the s.c. xenograft model, there were 25 genes whose expression was modified by radiation in both U87 and U251 cells. However, for U87 and U251 cells irradiated as i.c. xenografts, there were 729 genes (647 unique and named) commonly affected in both cell types (Supplementary Table 1A and B), which corresponds to a >40% overlap in the genes whose expression was modulated by radiation. The false discovery rate (11) for the commonly affected genes in the i.c. xenografts was 0.22, indicating that more than three quarters of the genes were likely to be true outliers (10). This similarity between the two glioma cell lines after i.c. irradiation suggests that the orthotopic environment not only modifies the number and types of genes affected but also reduces the cell type specificity of radiation-induced gene expression. These results are

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Figure 3. Genes affected in both U87 and U251 cells by radiation. Data in Figs. 1 and 2 were used to identify genes whose expression was changed by ≥2-fold under (A) tissue culture, (B) s.c., or (C) i.c. growth conditions. Yellow circles, exclusive to U87 cells; green circles, exclusive to the U251 cells; light green overlap, genes common to both cell lines.

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5 http://gostat.wehi.edu.au.
Figure 4. Biological processes and cellular components of the genes commonly affected in U87 and U251 cells by radiation under i.c. growth conditions. The genes whose expression was increased by radiation by ≥2-fold in both glioma lines grown under i.c. growth conditions were subjected to GOstat analysis. The tree diagram represents those Gene Ontology pathways that are statistically overrepresented under (A) biological process or (B) cellular component. The numbers of genes distributed to each pathway are listed in parenthesis to the right of each pathway. *, \( P < 0.01 \) and **, \( P < 0.001 \) (pathways that were statistically overrepresented).
consistent with our previous study showing that gene expression profiles for untreated U87 and U251 cells become similar when grown i.c. (5). Moreover, these data suggest that whereas genotype may be the overwhelming determinant for cells in tissue culture, under i.c. conditions, the normal brain environment plays a significant role in regulating the genes affected by radiation. Thus, taking into account such environmental influences will likely be critical in defining the putative functional significance of radiation-induced changes in gene expression.

The pathway analysis tool GOstat was used to determine whether the 729 common genes affected after irradiation of U87 and U251 cells grown under i.c. conditions corresponded to specific biological processes (12). GOstat distributes genes into biological processes and cellular components corresponding to Gene Ontology pathways (13), which are then organized in hierarchical clusters with the most general function at the primary node and more specific functions at each subsequent node. The number of genes expected to occur randomly in each pathway is compared with the actual distribution of genes in the sample set, which results in a list of biological pathways and cellular components that are statistically overrepresented in a given list of genes. For GOstat analysis, the commonly affected genes in i.c. U87 and U251 cells were divided into those whose expression was increased and those whose expression was decreased after irradiation. As shown in Fig. 4A, in the general, the Gene Ontology category of biological process, subgroup cellular process, was statistically overrepresented by genes whose expression was increased after irradiation; the genes within cellular process (332 genes) then primarily segregated into cellular physiologic process or metabolism. The more specific processes/functions within these two subnodes were pathways related to protein metabolism (112 genes) or biosynthesis (37 genes), RNA metabolism (21 genes) or biosynthesis (9 genes), and cell death (21 genes). In Fig. 4B, the subgroups within the Gene Ontology cellular component category [endoplasmic reticulum (23 genes), ribosome (24 genes), and ribonucleoprotein complex (32 genes)] were all statistically overrepresented. As there are over 4,000 Gene Ontology pathway annotations representing diverse biological processes, the significant overrepresentation of the above pathways suggests that the induction of gene expression after irradiation of i.c. glioma cells is not a series of random events but a component of the processes and events comprising tumor radioresponse. Interestingly, there were no pathways statistically overrepresented by the genes whose expression was decreased after irradiation.

Clearly, additional studies are required to address the radiobiological significance of the specific genes or gene sets induced after irradiation of i.c. grown glioma cells. However, the data presented indicate that environmental conditions play a critical role in determining the susceptibility of glioma cells to radiation-induced changes in gene expression. Moreover, the similarity in gene expression changes detected after irradiation of U87 and U251 cells grown i.c. suggests that this orthotropic condition mediates the activation of a general regulatory process not operative in tissue culture (14, 15). It will be of interest to determine the effect of the normal brain milieu on radiation-induced gene expression in other tumor histologies, particularly those that metastasize to the central nervous system. In addition, whether the orthotropic growth of other tumor types modulates their susceptibility to radiation-induced gene expression remains to be determined. However, at least for gliomas, these results suggest that orthotropic conditions may aid in defining the molecular components mediating radioresponse thus providing potential targets for radiosensitization, although a direct comparison to the microarray profiles of tumors from patients with gliomas undergoing radiotherapy would further validate this analysis.

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