Specific Gene Alterations in Radiation-Induced Tumorigenesis

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Abstract

To identify a set of genes involved in the development of radiation-induced tumorigenesis, we used DNA microarrays consisting of 1,176 mouse genes and compared expression profiles of radioresistant cells, designated NIH3T3-R#1 and –R#4. These cells were tumorigenic in a nude mouse grafting system, as compared to the parental NIH3T3 cells. Expressions of MDM2, CDK6 and CDC25B were found to increase more than 3-fold. Entactin protein levels were downregulated in NIH3T3-R#1 and –R#4 cells. Changes in expression genes were confirmed by reverse transcription-PCR or western blotting. When these genes were transfected to NIH3T3 cells, the CDC25B and MDM2 overexpressing NIH3T3 cells showed radioresistance, while

CDK6 overexpressing cells did not. In the case of entactin overexpressing NIH3T3-R#1 or R-#4 cells were still radioresistant. Furthermore, the CDC25B and MDM2 overexpressing cells grafted to nude mice, were tumorigenic. NIH3T3-R#1 and R#4 cells showed increased radiation-induced apoptosis, accompanied by faster growth rate, rather than and earlier radiation-induced G2/M phase arrest, suggesting that the radioresistance of NIH3T3-R#1 and R#4 cells was due to faster growth rate, rather than induction of apoptosis. In the case of MDM2 and CDC25B overexpressing cells, similar phenomena, such as increased apoptosis and faster growth rate, were shown. The above results, therefore, demonstrate involvement of CDC25B and MDM2 overexpression in radiation-induced tumorigenesis and provide novel targets for detection of radiation-induced carcinogenesis.

Introduction

Ionizing radiation is a well-established environmental mutagen and carcinogen. Several *in vitro* studies have shown that ionizing radiation produces a variety of genetic lesions, including deletions, rearrangements and point mutations. There exists a clear dose-response interrelationship among radiation-induced cell inactivation, chromosomal rearrangements, and mutagenesis. A question arises whether such interrelationships are valuable in understanding mechanisms of radiation carcinogenesis. There is an extensive research effort directed towards developing *in vitro* cellular systems that can describe events associated with radiation induced oncogenic transformation. In some established cell lines, neoplastic transformation can be induced by various agents, including some chemicals and radiation, and such transformed cells can induce tumors when grafted to the animal from which they were originally derived, or when transplanted into immunologically compatible hosts. Transformed cells *in vitro* usually display characteristics such as loss of anchorage dependence and contact inhibition, and changes at

DNA level. However, the exact mechanisms and the genes involved are not well characterized.

Those mechanisms have been under intensive study for the last two decades. At the molecular level, activation of oncogenes and inactivation of tumor suppressor genes have been known to be involved in radiation-induced carcinogenesis, together with abrogation of the DNA mismatch repair systems. Nevertheless, the exact mechanism of how these genetic alterations bring about the development and progression of radiation carcinogenesis remains largely unclear. To further complicate the picture, accumulation of mutant genes in neoplasia tends to be accompanied by other genetic and epigenetic changes, including loss of heterozygosity, inactivation of important genes by methylation or loss of imprinting, and gene amplification, all of which can alter gene expression profiles. Therefore, genome-wide monitoring of gene expression is of great importance, if we are to delineate the numerous and diverse events associated with carcinogenesis. In the present study, using microarray analysis, we identified genes from radioresistant cell lines that showed tumorigenesis in a nude-mouse grafting system.

Results

Selection of radiation resistant clones from NIH3T3 cells

NIH3T3 cells were irradiated twice per week for 6 months with 4Gy and 7 resistant clones were selected. When an *in vitro* clonogenic survival assay was performed, NIH3T3-R#1 and –R#4 cells showed the most radioresistance, whereas the rest of clones showed only slightly higher radioresistance than parental NIH3T3 cells. When cellular growth after radiation was examined by the trypan blue dye exclusion method, NIH3T3-R#1 and –R#4 cells showed increased growth rate by radiation, when compared to the control NIH3T3 cells.

Tumorigenesis of radiation-resistant clones when grafted into nude mice

To elucidate the relationship between cells surviving after repeated radiation treatments and

tumorigenesis, control NIH3T3, NIH3T3-R#1, and –R#4 cells were grafted into nude mice. Examination of tumorigenesis revealed that the parental NIH3T3 cells did not show any tumors, while the resistant clones, NIH3T3-R#1 and –R#4, induced tumors. Tumor burden in NIH3T3-R#1 and –R#4 after 6 weeks of grafting. Histological examination also suggested that tumor sections of NIH3T3-R#1 and –R#4 showed characteristic tumor morphology; clear cells, increased apoptotic body, giant cells (polynuclei cells) and necrosis.

Altered gene expression, revealed by microarray, in radiation-resistant clones

We used cDNA expression array hybridization to identify genes that were differentially expressed in radioresistant NIH3T3 clones. For the comparison of the autoradiographic intensities, two radioresistant R#1 and R#4 clones were selected for cDNA array analysis. When we selected the genes which showed expression alterations in both radioresistance R#1 and R#4 clones, cdk6, mdm2, cdc25B and entactin (3.5 fold increase, 4.3 fold increase, 3.7 fold increase, and 1.6 fold decrease, respectively). After cDNA expression array hybridization, to further validate the approach by cDNA array, we performed RT-PCR analysis. As seen in Fig 3A, 4 genes including cdk6, cdc25B, mdm-2, and nidogen-1 (entactin) genes were selected. Western blot analysis for MDM2. CDC25B and CDK6 used well consistent with the results of cDNA hybridization array and RT-PCR. Furthermore, *In situ* immunocytochemical analysis revealed increased expressions of CDK6, MDM-2 and CDC25B proteins; CDK6 and CDC25B proteins were slightly expressed in the cytosol of normal NIH3T3 cells, but increased amount of proteins were detected in the resistant clones. In the case of MDM-2, nuclear expression was found in the normal NIH3T3 cells and increased nuclear protein expression was observed in the resistant clones.

MDM2 or CDC25B overexpression in parent NIH3T3 cells induced tumorigenesis when grafted to nude mice

Whether radioresistance of MDM2 or CDC25B overexpressed cells was involved in tumorigenesis, these cells were grafted to nude mice. After 6 weeks, NIN3T3-R#1 and –R#4 cells showed tumorigenesis, while the parent NIH3T3 cells did not. In addition, 3 clones of each MDM2 or CDC25B overexpressed also showed tumorigenesis, thus suggesting that MDM2 or CDC25B overexpression in NIH3T3-R#1 and –R#4 was involved in NIH3T3-R#1 and –R#4-mediated tumorigenesis. When grafted, tumorigenesis was not observed in CDK6 overexpressing NIH3T3 cells.

Discussion

In the present study, we identified 4 genes, CDK6, CDC25B, MDM2 and entactin, whose expression was altered in radiation-resistant NIH3T3 cells. Since these cells induced tumorigenesis, it is highly likely that alteration of these genes are involved in tumor generation.

There are very few published studies on radiation-induced transformation and tumorigenesis. Hei *et. al*. reported that viral antigen immortalized human bronchial epithelial cells were transformed to the malignant state by a single exposure to radon-simulated alpha-particles. Similarly, it has been demonstrated that human keratinocytes, immortalized by a combination of DNA tumor viral antigens, were transformed by two or more exposures to gamma radiation or by a single exposure to fission neutrons. Riches *et. al.* reported that malignant transformation of SV40-virus immortalized human thyroid cells after a single exposure to gamma irradiation, and Wazer *et. al*. successfully transformed a finite life span human mammary cell lines to malignancy by repeated exposures to ionizing radiation. In our system, we selected 2 radioresistant NIH3T3 clones (NIH3T3-R#1 and –R#4) after repeated irradiation. Parental NIH3T3 cells did not show any tumorigenicity when grafted into nude mice, while two radioresistant clones did, indicating that repeated radiation induced gene alterations, resulted in

radiation-induced tumorigenesis.

Gene expression profiling can significantly increase our understanding of the mechanisms and pathways that regulate the transition from normal to tumor state. The advantage of cDNA microarray technology is that it is possible to analyze thousands of genes simultaneously. The 7 genes, which were five fold overexpression in both NIH3T3-R#1 and –R#4 cells and those were MDM2, CDK6, CDC25B, secreted phosphoprotein, procollagen type III, CD-2 and associated protein. On the other hand, entactin was 2 fold underexpressed in both of the resistant cells, compared to parental NIH3T3 cells. When MDM2, CDK6, or CDC25B were overexpressed in NIH3T3 parent cells and entactin in tumorigenic clones of NIH3T3-R#1 and –R#4, radioresistance was found only in MDM2 and CDC25B overexpressing cells. In the cases of CDK6 and entactin, there was no difference found in clonogenic cell survival assay. Next, experiments were performed to answer the question of whether MDM2 and CDC25B overexpression was responsible for tumorigenesis. Thus, overexpressing MDM2 or CDC25B cells were grafted into nude mice and tumorigenesis was examined. After 6 weeks of $1x10^6$ cells per mouse, MDM2 and CDC25B overexpressing cells showed tumorigenesis as much as the level of tumorigenic radioresistant cells (NIH3T3-R#1 and –R#4).

In conclusion, even though we do not know exactly how these gene expressions are altered by radiation, we herein attempted best to characterize the genes which are responsible for radiation-resistance and radiation-induced tumorigenesis. Further studies are needed to elucidate the cellular characterization of these resistant cell lines as well as to clarify the potential direct relationship between these four genes, radiation-resistance, and tumorigenesis using an overexpression system. However, our data strongly implicate novel targets for detection of radiation-induced carcinogenesis.

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