Inhibition of Potential Lethal Damage Repair and Related Gene Expression after Carbon-ion Beam Irradiation to Human Lung Cancer Grown in Nude Mice

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Using cultured and nude mouse tumor cells (IA) derived from a human lung cancer, we previously demonstrated their radiosensitivity by focusing attention on the dynamics of tumor clonogens and the early and rapid survival recovery (potential lethal damage repair: PLD repair) occurring after X-ray irradiation. To the authors’ knowledge, this is the first study demonstrating gene expression in association with PLD repair after carbon-ion beam or X-ray irradiation to cancer cells. In this study we tried to detect the mechanism of DNA damage and repair of the clonogens after X-ray or carbon-ion beam irradiation. At first, colony assay method was performed after irradiation of 12 Gy of X-ray or 5 Gy of carbon-ion beam to compare the time dependent cell survival of the IA cells after each irradiation pass. Second, to search the genes causing PLD repair after irradiation of X-ray or carbon-ion beam, we evaluated gene expressions by using semi-quantitative RT-PCR with the selected 34 genes reportedly related to DNA repair. The intervals from the irradiation were 0, 6, 12 and 24 hr for colony assay method, and 0, 3, 18 hr for RT-PCR method. From the result of survival assays, significant PLD repair was not observed in carbon-ion beam as compared to X-ray irradiation. The results of RT-PCR were as follows. The gene showing significantly higher expressions after X-ray irradiation than after carbon-ion beam irradiation was PCNA. The genes showing significantly lower expressions after X-ray irradiation rather than after carbon-ion beam irradiation were RAD50, BRCA1, MRE11A, XRCC3, CHEK1, MLH1, CCNB1, CCNB2 and LIG4. We conclude that PCNA could be a likely candidate gene for PLD repair.

INTRODUCTION

A clinical trial of heavy-ion radiotherapy was started in June 1994 at the Heavy-ion Medical Accelerator in Chiba (HIMAC). During the past 9 years, over 1600 patients have been treated with carbon-ion beam, and encouraging results have been obtained for several cancers. Heavy-ion beam has several advantages compared to conventional photon therapy because of their accurate dose localization and radiobiological features of high-LET beam.\textsuperscript{1,2} Moreover, many experimental studies are also undertaken in several laboratories to investigate the radiobiological features of heavy-ion beams generated by HIMAC concerning, for example, the relative biological effects, apoptosis, repopulation, reoxygenation and PLD.\textsuperscript{2,3} High-LET radiation is more effective than low-LET, X- or γ-radiation in inducing biological damage.\textsuperscript{4} The Bragg-peak of an accelerated charged particle beam provides superior dose localization in the critical cancer tissue. High-LET radiation also gives a higher RBE value for cell killing,\textsuperscript{5} a reduced oxygen effect\textsuperscript{5} and a reduced dependence on the cell-cycle,\textsuperscript{6} and its effects are superior to those achieved with low-LET radiation in cancer treatment.

Reports in the 1970s claimed that PLD repair had been measured and defined operationally as an increase in survival after a single radiation dose when the cells are exposed to various post-irradiation environmental conditions.\textsuperscript{7} Reports published in 1980 indicated that PLD repair involved steps at the DNA level inhibiting PLD repair by β-D-Arabinofuranosyladenine whose function consists of the selective inhibition of DNA synthesis via DNA polymerases.\textsuperscript{8,9} An increased understanding of the mechanisms of PLD repair might provide tools to selectively interfere with repair
pathways and to customize treatment for individual patients. During the past decade, bio-molecular techniques have been developed, but there are only a few studies on the molecular biological effect and gene expression in association with PLD repair.6–12

Using cultured and nude mouse tumor cells (IA) derived from human lung cancer, we previously demonstrated their radiosensitivity by focusing attention on the dynamics of tumor clonogens and the early and rapid survival recovery (PLD repair) occurring after X-ray irradiation.13 To the authors’ knowledge, this is the first study demonstrating gene expression in association with PLD repair after carbon-ion beam or X-ray irradiation to cancer cells.

MATERIALS AND METHODS

Cells and Tumor
A human lung cancer cell line grown in a culture and in nude mice was used as the tumor in our present experiments. These cancer cells had been derived from a metastatic skin cancer in a patient with primary lung cancer, diagnosed on histological evidence as large cell carcinoma. The cells on a Falcon plastic dish in an F10 medium supplemented with 10% fetal calf medium and antibiotics (PC, SM) were grown at a doubling time of 24 hr in a 5% CO2 chamber at 37°C. The BALB/c-nu/nu (a thymic nude mouse strain) mice were fed on a sterilized commercial pellet diet (MB-1, Funabashi Co, Ltd, Chiba) and water under specific pathogen-free (SPF) conditions in our Institute. Animal experimental protocols for ethics were approved by the NIRS Institutional Animal Care and Use Committee, and were performed according to the institutional guidelines. For the formation of a tumor, 2–3 × 106 cells applied in a single cell suspension of 50 μl were implanted into the back of the 4 week old male nude mice with a microsyringe, previously primed with Cs137 irradiation at 360cGy. A measurable tumor appeared around 12 days later and continued to grow steadily. The size reached from about 3 mm to 15 mm in average diameter. The tumor clonogens and the early and rapid survival recovery radiosensitivity by focusing attention on the dynamics of tumor clonogens and the early and rapid survival recovery (PLD repair) occurring after X-ray irradiation.13) To the authors’ knowledge, this is the first study demonstrating gene expression in association with PLD repair after carbon-ion beam or X-ray irradiation to cancer cells.

Colony assay method
To measure cell survival, growing culture cells were dispersed with 2.5% trypsin solution for 5 minutes at 37°C. The mice were killed by cervical dissection. Immediately after a whole tumor had been taken out it was thoroughly minced to a paste with sharp scissors taking care to exclude all bloody and fibrous masses. The paste was dissolved in a complete medium by repeated pipetting, and mechanically dispersed by passing the solution twice through a set of metal mesh screens, the first one having a pore size of 300 μ and the second one a pore size of 50 μ. The single cell suspension thus obtained from the culture cells or the tumor in the nude mice, were stained with 4% Erythrosin B solution. The viable cells were counted using a hemocytometer, adjusted in cell number and plated onto triplicate dishes to grow a colony for 12 days in 5% CO2 incubator. A cell population with more than 50 cells was considered a surviving colony. For the culture cells growing at an exponential rate, the percentage of stained cells was 2.4% ± 0.5% and the plating efficiency was 43.9 ± 7.1%. For the nude mouse-derived cells, the former was 40.2 ± 10.9% and the latter was 28 ± 6.1%. The total clonogenic cell (clonogen) count was obtained for each mouse in each experiment. The number of total viable tumor cells was determined by measuring the percentage of cells not stainable with Erythrosin B × plating.
| Gene                        | Accession No. | Forward primer (5'-3')       | Reverse primer (5'-3')       |
|-----------------------------|---------------|------------------------------|------------------------------|
| **Homologous recombination** |               |                              |                              |
| RAD50                       | NM_005732     | CTACCGAGTGGTGATGCTGA         | CCACAGTTGAGGCAGAAGC          |
| RAD51C                      | NM_058216     | GCTTAGCCCAAGGAAAGATGATGCGA  | AACAAGCAGGGGCTAGGTTCTCAGA   |
| RAD51L1                     | NM_002877     | ATCGCTTCTTGTGGCCACA         | TTCCATGACTTGAAGCTGATCTACA   |
| RAD51L3                     | NM_002878     | CCAACCACATACAAGGGATGACAG    | TCGATGGTGCAGCCAGAAGATC      |
| RAD52                       | NM_002879     | GACGCGACAGCACTCCTGTAAC      | AGTCAGACCCATCCTTGACAAG      |
| RAD54L                      | NM_003579     | GAAGCCCTGAGAAGTGCACAA      | CTTGGCTCTGCTTCTTAATTG       |
| BRCA1                       | NM_007294     | CAAGAACCGGTTTCCAAAGA       | TGAAATAATCCCAGGAGAAGCT      |
| BRCA2                       | NM_000059     | TTCATGGAGCAGAAGTGGTG       | GGGTCAGAAGGAAATCCAAGG       |
| MRE11A                      | NM_005590     | CAGACATCTGGTCTGAGCAGTC     | AGTAGTGACATTTGCGAGGAGG     |
| PCNA                        | M15796/N24235 | AGGCTCTGGAGCCGAAGAC        | TGGCAACAGCCGCGC            |
| XRCC3                       | NM_005432     | CTCAGAAGCTCGGATTGAG        | AGACAGCCTAGGGGAGGCT        |
| **DNA damage checkpoint pathway/cell cycle checkpoint pathway** |               |                              |                              |
| RAD1                        | NM_002853     | GCTCCCTGGAGAACATTTTCTG     | GAAGACTTCCCTGCAATTTCCA     |
| RAD9                        | NM_004584     | TACTCTTGGCGTCTGACTTG       | GGCCTCCCTCCTCAATCTAA       |
| RAD17                       | NM_133341.1   | CAGCTATGGCATACTCTTCG       | GTCAGGGCTCTCAATTTCAA       |
| CHEK1                       | NM_001279.2   | TCTGCTAGATATGGTGACATTG     | TCTACATATTCTCAACAAATTC     |
| CHEK2                       | NM_007194.2   | ATCCAAAGGCAGGTTCATG       | TGGGGTAGAGCTGTTGGGATC       |
| HUS1                        | NM_004507     | ATCGCTITGGCTGCTGCTGCA     | CAGACATCTGGTCTGAGCAGTC     |
| TP53                        | U94788        | CCATCTCACCATCATCACA       | GTGACCGCTCCTCTTTTCTG       |
| TP53NIP1                    | NM_033285     | TGATTCATAGTGGTGACATTG     | AAAATGCACCAAAACCAAT        |
| CDKN1A                      | L25610        | ACCCTCTCTATCCCCACCA       | TGACTCCTGTGTGCTGCTG        |
| WEE1                        | NM_003390     | AGACCTTTAGCTAAGGACT       | TGGCCTATGTCGTTTTCTG        |
| CDC25B                      | M18934        | TGGATGCGACAGGCTTTG       | GCTGACGGTCTATGCTCA         |
| **Mismatch excision repair** |               |                              |                              |
| MLH1                        | NM_000249     | TATCTTCTTGACTAGGCTACTGA   | GAAGGCAAGGCGACAGGCTG       |
| MSH2                        | NM_000251     | AGCCCTGGAACATTGAGGAG     | AGGGCATTTGTTTCACCTTG       |
| **Non-homologous end-joining** |             |                              |                              |
| LIG4                        | NM_002312     | TCTGAAAAAGAAGTTGGGTAACCTGA | CTGCACGATCAAGGGCTTTC       |
| XRCC4                       | NM_003401     | CAGACCTGCTACATGTTGATG    | TGCAGGCTTCGTCAACTCA         |
| **Genes defective in diseases associated with sensitivity to DNA damaging agents** |               |                              |                              |
| ATM                         | NM_138292     | AGAGCCGCGGAGATGAACT       | AAGACAGCTGTCAGTCTTGGTTG     |
| FANCC                       | X66893        | GATTTAACACTTAAAGGCGAAGATG | TAAATGTTGAGGACAAACCAATGG   |
| **OTEHRS**                  |               |                              |                              |
| ABL1                        | NM_005157     | ACTGCTCTGTCTCCATGAGG      | TGGCACGCGCAGCTAAGAAA       |
| ABL2                        | NM_005158     | AACTCAGCTGCTGGAGAGCTA    | GCTAACCTGTCACCAATCGA       |
| CCNB1                       | NM_031966     | TCAAGGACTTACAAGACAGATGAC   | GCCACAGCCCTGGCTAAT         |
| CCNB2                       | NM_004701     | AGCTCTTGTCTTGTGCTCTC    | TGGGCTGACAGCTTCCACT       |
| RFC2                        | NM_002914     | GGTGCGACAGTGGAGCAGAG     | GACAGGCTCATGTTGCTCAGG      |
| RFC5                        | NM_007370     | CATCCTGGACTGAGTGTTGA    | TCGAAGACTGAGATGGAGAGAGTA   |
RNA extraction and semi-quantitative RT-PCR for DNA repair related gene expression

Total RNA was isolated from irradiated nude mice using RNeasy Mini Kit (QIAGEN, Tokyo, Japan). The reverse transcriptase reaction was performed using 100 ng of total RNA, oligo(dT) and RT-PCR Systems (Invitrogen, Tokyo, Japan) according to manufacturer’s instructions. DNA contamination was excluded by using DNaseI. PCR was carried out in MX3000P (STRATAGENE, Tokyo, Japan) using the SYBR Premix Ex Taq (Perfect Real Time) according to manufacturer’s instructions (TAKARA Bio, Shiga, Japan). The primers for β-actin were forward: 5’-AAGTACTCCGTGTGGATCGGC, reverse: 5’-AGCATTTGGCCTGGGCGA. The conditions for PCR were as follows: initial denaturation at 95°C for 10 seconds, step of 50 cycles of denaturation at 95°C for 10 seconds, annealing/extension step at 60°C for 30 seconds, followed by 1 minute at 95°C. To determine the quantity of the target gene-specific transcripts present in irradiated cells relative to non-irradiated ones, their respective Ct values were first normalized by subtracting the Ct value obtained from the β-actin control (ΔCt = Ct’ target – Ct’ control). The concentration of gene-specific mRNA in irradiated cells relative to non-irradiated cells was calculated by subtracting the normalized Ct values obtained for non-irradiated cells from those obtained from irradiated samples (ΔΔCt = ΔCt’ treated – ΔCt’ untreated) and the relative concentration was determined (2−ΔΔCt). cDNA quality was tested by RT-PCR using human β-actin primer. RT-PCR primer sequences for the individual genes and β-actin were selected with the aid of the Primer 3 program.

Table 1 shows primer list for RT-PCR reactions to obtain the specific gene expression rate of the selected 34 genes reported to be related to DNA repair. The genes in the list were preferentially selected for the rapid pathway for DNA double strand breaks.

Statistical analysis

Statistical analysis was performed with StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, USA). Bonferroni-Dunn test was performed to test differences in the survival fraction of colony assay experiments and in the amount of gene expression between carbon-ion beam and X-ray irradiation. A P-value less than 0.05 were regarded as significant.

RESULTS

Growth dynamics of clonogens after irradiation of carbon-ion beam or X-ray

Colony assay experiments were performed to compare the differences in terms of the clonogenic growth dynamics of X-ray and carbon-ion beam irradiation (Fig. 1). Clonogenic cells were inactivated immediately, but in turn, significantly increased during 24 hr (p < 0.05) after X-ray irradiation, whereas within 24 hr after carbon-ion beam irradiation, cells showed a significantly smaller increase rather than after X-ray irradiation. In relation to the growth dynamics of clonogens without irradiation, clonogenic cells were reported to increase over time.13 As shown in Fig. 1, a 5 Gy dose of carbon-ion beam and a 12 Gy dose of X-ray were almost exactly identical in terms of their respective cytocidal effect.10 It therefore seemed reasonable to conclude that an early significant increase in the survival fraction within 24 hr occurred after X-ray irradiation, whereas the increase in survival was inhibited after carbon-ion beam irradiation. However such an increase in survival fraction after X-ray and carbon-ion beam irradiation might indicate PLD repair.
Relative gene expression rate after irradiation of carbon-ion beam or X-ray

Figure 2 shows the results for the relative gene expression obtained with semi-quantitative RT-PCR. To elucidate the mechanism of rapid increase in the survival fraction occurring 24 hr after X-ray irradiation only, we compared the gene expression rate after X-ray with that after carbon-ion beam irradiation. We performed semi-quantitative RT-PCR of the selected 34 genes at the 0, 3 and 18 hr time points after irradiation with carbon-ion beam or X-ray.

At 0 and 3 hr after irradiation, no gene showed a significantly different relative gene expression rate after X-ray exposure than that after carbon-ion beam. At 18 hr after irradiation, a time point at which PLD repair is thought to be stable from the result of this colony assay experiments, gene expressions of PCNA (p = 0.0002) after X-ray exposure was significantly higher than those after carbon-ion beam. In contrast, gene expressions of RAD50 (p = 0.0017), BRCA1 (p = 0.0002), MRE11A (p = 0.0002), XRCC3 (p < 0.0001), CHEK1 (p < 0.0001), MLH1 (p < 0.0001), CCNB1 (p = 0.0003), CCNB2 (p = 0.0028) and LIG4 (p = 0.0007) after X-ray were significantly lower than those after carbon-ion beam (Fig. 2).

The IA cells used in this experiment have point mutation

Fig. 2. Relative gene expression rates18 hr after 12 Gy of X-ray (gray columns) and 5 Gy of carbon-ion beam (white columns) irradiation. This figure shows only the genes that exhibited significant differences between X-ray and carbon-ion beam irradiation. Gene expressions of PCNA after X-ray exposure were significantly higher than those after carbon-ion beam. In contrast, gene expressions of RAD50, BRCA1, MRE11A, XRCC3, CHEK1, MLH1, CCNB1, CCNB2 and LIG4 after X-ray were significantly lower than those after carbon-ion beam. These expression rates were determined for each gene by dividing with the expression rate of the control tumor. Each value shown is the mean of three independent experiments, and error bars represent the S. D. Data were analyzed using the Bonferroni-Dunn test.
in exon6 (codon192) of p53, and our results for the time dependent gene expression rates of TP53 and its downstream effector, CDKN1A, a protein whose activation kinetics closely follows p53 activation, did not significantly vary.

**DISCUSSION**

We demonstrated that a small increase in survival fraction occurs within 24 hr after carbon-ion beam exposure and a significant increase after X-ray irradiation in the colony assay method. This is an initial study designed to list the set of genes involved in the unknown mechanism of the early and rapid increase in the survival fraction following X-ray irradiation in comparison with carbon-ion beam, and attention was thus focused on the relationship between the phenomena and the selective DNA repair related genes in the RT-PCR method.

PLD is the component of radiation damage that can be modified depending on the post-irradiation conditions. Under ordinary circumstances, PLD causes cell death. Changing cellular growth conditions or the microenvironment of cells influences the expression of PLD or its repair, and thereby influences radiation sensitivity. PLD repair is favored by conditions that maintain cells without encouraging or allowing them to divide. Conditions found in solid tumors, where regions of the tumor may be far from the blood vessels and show low glucose, low oxygen, low extracellular pH, and high concentrations of cellular waste products, may prevent cells from proliferating, and thereby promote PLD repair.\(^{10}\)

Whether the PLD repair depends on LET has not been fully investigated. From the result of this survival assay, PLD repair was clearly found after irradiation of X-ray with low-LET in the IA cells. A possible explanation may lie in the behavior of the damaged cells, which are fated to die. This may be one of the major causes for rapid increase in survival fraction after X-ray irradiation. The cells that sustain low-LET type lethal damage continue to live, and then fail to divide. Yet it is also possible that these cells with fatal, but relatively slight damage may divide to produce daughter cells.\(^{17}\) William et al. have shown that no repair of PLD was observed in the Lewis lung carcinoma after neutron treatment.\(^{18}\) The damage brought about by high-LET radiation is usually so dense and complicated that the damaged cells cannot be repairable.

Recently, an immediate accumulation of TP53 and CDKN1A protein 3 to 6 hr after exposure to X-ray or carbon-ion beams has been observed in human fibroblasts in vitro.\(^{39}\) In this study, no gene at the early time points, e.g., 0 hr and 3 hr after irradiation to the human lung cancer grown in nude mice in vivo showed neither a significant increase nor a decrease in relative gene expression rate. We assume that the differences in our results might be due to in vivo circumstances.

As to the role of p53 in PLD, some studies have demonstrated that PLD repair was dependent on a functional G1 arrest mediated by the wild-type p53 function.\(^{10,20,21}\) However, it has recently been reported that PLD repair might be present without a functional p53 in the human glioblastoma cell line Gli-06.\(^{12}\) With the IA cells having point mutation at p53, our result showed no significant differences in the gene expression rates of TP53, TP53INP1 and CDKN1A between carbon-ion beam and X-ray. It might mean the possibility of the existence of PLD repair pathway without a functional p53 in this IA cells.

Although Geoff W. Birrell et al. have reported that transcriptional response of yeast to DNA-damaging agents: ionizing radiation, UV radiation, and exposure to cisplatin or to hydrogenperoxide does not identify the genes that protect against these agents;\(^{22}\) our results of IA cells grown in nude mice listed some genes whose expressions changed significantly after different type of irradiation. Figure 2 shows that the gene expressions of only PCNA after X-ray were significantly higher than those after carbon-ion beam, and that the gene expressions of RAD50, BRCA1, MRE11A, XRCC3, CHEK1, MLH1, CCNB1, CCNB2 and LIG4 after X-ray were significantly lower than after carbon-ion beam. We were able to explain that as a result of gene expression changes X-ray induced damage may be repaired completely by 18 hr while the damage is repaired to a lesser extent in response to carbon-ion beam. In other words, the increase in gene expression may be a consequence of unrepaired damage induced by carbon-ion beam; thus, PCNA which showed higher gene expression after X-ray could be meaningful to PLD repair.

The mechanism underlying PLD repair in IA cells is still unclear, and further bio-molecular studies with comprehensive genes including unknown genes are required to determine the clinical relevance of this finding.

In conclusion, our results showed significant differences between X-ray and carbon-ion beam irradiation for 10 genes: PCNA (at 18 hr), RAD50 (at 18 hr), BRCA1 (at 18 hr), MRE11A (at 18 hr), XRCC3 (at 18 hr), CHEK1 (at 18 hr), MLH1 (at 18 hr), CCNB1 (at 18 hr), CCNB2 (at 18 hr) and LIG4 (at 18 hr). We conclude that PCNA could be a likely candidate gene for PLD repair.

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