



The Effects of Hydrogen Peroxide and/or Radiation on the Survival of Clinically Relevant Radioresistant Cells

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Abstract

Background: Radiation therapy is a highly cost-effective treatment for cancer, but the existence of radio-resistant cells remains the most critical obstacle in radiotherapy. We have been established clinically relevant radioresistant (CRR) cell lines by exposure to a stepwise increase of fractionated X-rays. We are trying to overcome the radio-resistance by analyzing the properties of these cells. In this study, we tried to evaluate the effects of hydrogen peroxide (H₂O₂) on the CRR cells because this can evaluate the efficacy of Kochi Oxydol-Radiation Therapy for Unresectable Carcinomas (KORTUC) that treats H₂O₂ before irradiation. We also established H₂O₂-resistant cells to compare the radiation and H₂O₂ resistant phenotype. **Materials and Methods:** We used human cancer cell lines derived from hepatoblastoma (HepG2), oral squamous cell carcinoma (SAS), and cervical cancer (HeLa). We established HepG2, SAS, and HeLa CRR cells and HepG2, SAS, and HeLa H₂O₂-resistant cells. To evaluate their sensitivity to radiation or H₂O₂, high-density survival assay, or WST assay was performed. CellROXTM was used to detect intracellular Reactive Oxygen Species (ROS). **Results:** CRR cells were resistant to H₂O₂-induced cell death but H₂O₂-resistant cells were not resistant to irradiation. This phenotype of CRR cells was irreversible. The intracellular ROS was increased in parental cells after H₂O₂ treatment for 3 h, but in CRR cells, no significant increase was observed. **Conclusion:** Fractionated X-ray exposure induces H₂O₂ resistance in CRR cells. Therefore, it is necessary to carry out cancer therapy such as KORTUC with the presence of these resistant cells in mind, and as the next stage, it would be necessary to investigate the appearance rate of these cells immediately and take countermeasures.

Keywords

radiation therapy, clinically relevant radioresistant cells, reactive oxygen species, cell death, H₂O₂ resistant cells

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Introduction

Radiation therapy is a highly cost-effective single-modality treatment for cancer. Ionizing radiation (i.e. X-rays or γ -rays) is commonly used for cancer treatment because it can pass through tissue, break chemical bonds, and remove electrons from atoms to become ionized. Cancer treatment utilizing fractionated radiation therapy has distinct advantages over a single administration of radiation because the former can increase the anticancer therapeutic effect and decrease the occurrence of side effects in normal tissues.¹ Empirically, in conventional fractionated radiation therapy, approximately 1.8-2 Gy of radiation is administered daily, resulting in 9-10 Gy administered per week, which amounts to approximately 54-60 Gy for

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a total of 6 weeks. Although radiation therapy may be curative in various cancer types, the resistance of cancer cells to radiation remains a major therapeutic problem.^{2,3} In order to destroy the radioresistant cells, chemotherapeutic agents are also used for cancer treatment (radio-chemotherapy).

One of the first events that occur after irradiation is the induction of DNA damage. Ionizing radiation can directly interact with critical cellular targets, such as DNA. This results in energy transfer, which induces atom ionization and subsequent biological changes (direct action). Additionally, ionizing radiation instantaneously induces the formation of water radiolysis products that contain reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide (H_2O_2), and hydroxyl radicals. These ROS exhibit high reactivity to a variety of cellular macromolecules, including DNA, lipids, and proteins. These free radicals can interact with macromolecules, such as DNA, and induce biological changes (indirect action). In the presence of O_2 , ionizing radiation converts hydroxyl radicals, superoxides, and organic radicals to H_2O_2 and organic hydroperoxides.⁴ H_2O_2 is converted into hydroxyl radicals by a Fenton-type reaction. The hydroxyl radicals induce cellular lesions which are similar to lesions induced by ionizing radiation.⁵ Hydroxyl radicals induce both chromosome breaks and the formation of numerous micronuclei in dividing cells.

In order to elucidate the mechanisms underlying tumor cell radioresistance, clinically relevant radioresistant (CRR) cell lines were developed by exposure to a stepwise increase of fractionated X-rays.⁶ CRR cells proliferated for more than a month when exposed to 2 Gy/day of X-rays. Recently, it was shown that CRR cells are resistant to H_2O_2 .⁷ Conversely, it is unknown whether H_2O_2 -resistant CRR cells are also resistant to X-rays. In this study, H_2O_2 -resistant cancer cell lines were developed to evaluate their sensitivity to radiation.

Ogawa et al. developed a unique radiosensitization treatment using a H_2O_2 solution (Oxydol)-soaked gauze referred to as Kochi Oxydol-Radiation Therapy for Unresectable Carcinomas (KORTUC I). KORTUC I was applied to superficially exposed and unresectable neoplasms, such as malignant melanoma and malignant fibrous histiocytoma (MFH). The highly radioresistant human osteosarcoma cell line, HS-Os-1 was also subjected to KORTUC.⁸ KORTUC I induces radiosensitization of radioresistant tumors by blocking antioxidative enzymes such as peroxidases, which results in local oxygen production. A new injectable radiosensitizer (KORTUC II) may convert radioresistant tumors into radiosensitive tumors.⁹ In this study, the effects of H_2O_2 on the radioresistance of CRR cells was evaluated, which may be extrapolated to evaluate the efficacy of KORTUC for cancer treatment.

Materials and Methods

Cell Culture

Human cancer cell lines derived from hepatoblastoma (HepG2), oral squamous cell carcinoma (SAS), and cervical cancer (HeLa) tissues were obtained from the Cell Resource

Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The CRR cells are referred to by their cell line name followed by an R to denote their radioresistance. The HepG2-8960-R and HepG2-R cells were independently established from HepG2 cells. The SAS-R and HeLa-R cells were derived from SAS and HeLa cells, respectively, by exposing the cells to gradually increasing X-ray doses.¹⁰ The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 5% fetal bovine serum (Gibco Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere at 37°C with 5% CO_2 . Exponentially growing cells were used in all of the experiments. X-ray irradiation was conducted with a dose of 1 Gy/min in a 150-KVp X-ray generator (MBR-1520 R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum and 0.1 mm copper. H_2O_2 -resistant cell lines were obtained utilizing a conditioning method. Briefly, parental cells were cultured in medium containing increased levels of H_2O_2 in a stepwise fashion. CRR cell lines were developed by exposing cells to a stepwise increase in fractionated radiation from 0.5 Gy/day to 2 Gy/day. We hypothesized that the H_2O_2 -resistant phenotype of CRR cells originated during the development of the CRR cell lines. In order to test this hypothesis, we obtained 2 types of cell lines. One cell line was exposed to 0.5 Gy/day \times 10 (total dose 5 Gy) of X-rays and another cell line was exposed to 0.5 Gy/day \times 10 + 1 Gy/day \times 10 (total dose 15 Gy) of X-rays. Both of these cell lines were assayed for their sensitivity to H_2O_2 utilizing a water-soluble tetrazolium salt (WST) assay.

High-Density Survival Assay

The sensitivity of cells to X-rays was determined utilizing a modified high-density survival (MHDS) assay as previously reported.¹¹

WST Assay

The effect of H_2O_2 on cell proliferation was monitored with a WST assay, which was conducted using the Cell Count Reagent SF (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's protocol. Briefly, 5×10^3 exponentially growing cells were plated in a 96 well plate 24 hours before the experiment. Next, the cells were incubated for 48 hours in RPMI 1640 medium containing H_2O_2 . Subsequently, 10 μ L of Cell Count Reagent SF was added to each well and the optical density of each well was determined with a Multiskan JX microplate reader (Thermo Fisher Scientific Inc., MA, USA). In order to determine the radiosensitizing effects of H_2O_2 in CRR cells, the cells were treated with H_2O_2 for 48 hours followed by exposure to 10 Gy of X-rays.

Intracellular ROS Assay

CRR cells were treated with or without 100 μ M of H_2O_2 and the intracellular ROS levels were determined using the

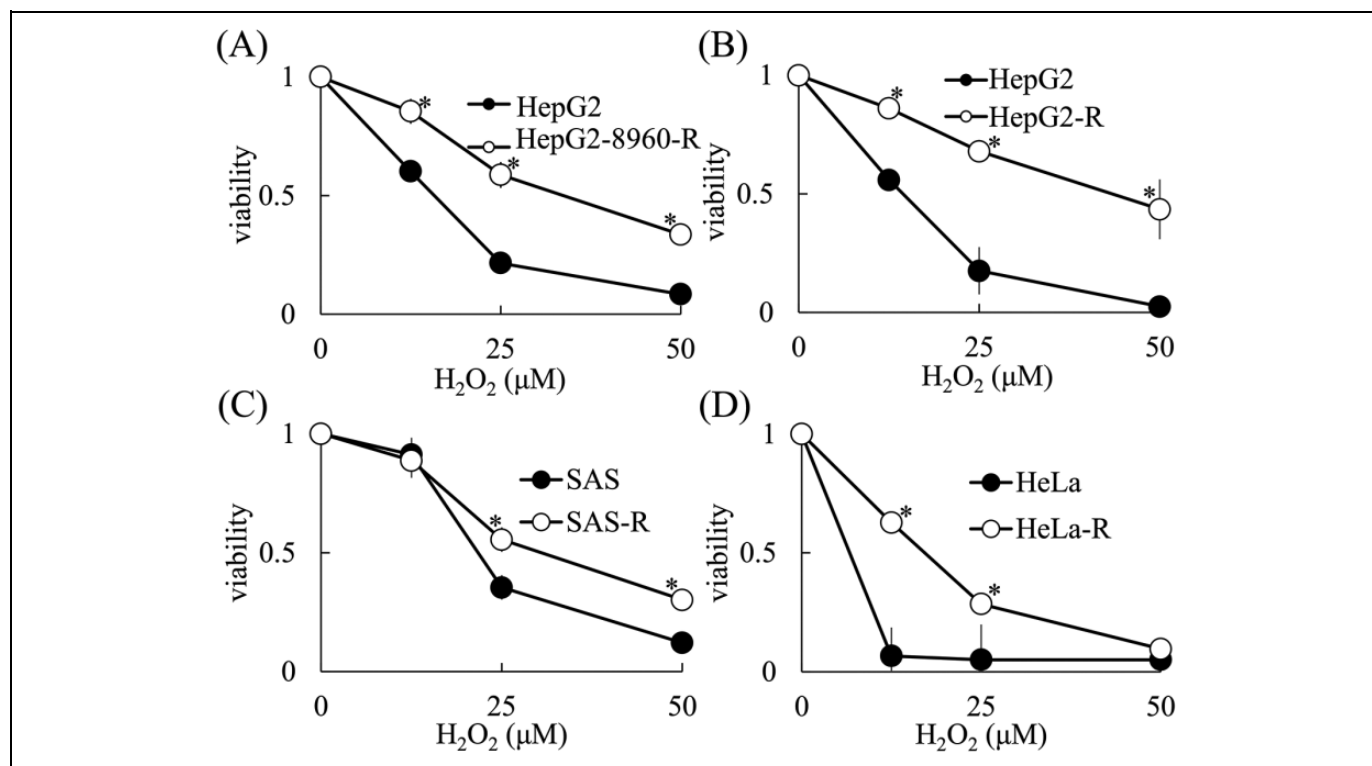


Figure 1. CRR cell lines are resistant to H₂O₂-induced cell death. Cells (5×10^3) were incubated with H₂O₂ for 48 hours and a WST assay was performed according to the manufacturer's protocol. A, H₂O₂ sensitivity of parental HepG2 and CRR HepG2-8960-R cells. B, H₂O₂ sensitivity of parental HepG2 and CRR HepG2-R cells. C, H₂O₂ sensitivity of parental SAS and CRR SAS-R cells. D, H₂O₂ sensitivity of parental HeLa and CRR HeLa-R cells. The data represent the mean \pm SD from 3 independent experiments and are presented relative to the control parental cell lines. *: $p < 0.05$ using Student's *t* test (vs. Parental cells).

CellROX™ Green Reagent (Thermo Fisher Scientific, Inc., PA, USA) according to the manufacturer's protocol. Fluorescent images of the cells were obtained using a BZ-8000 Fluorescence Microscope (KEYENCE, Osaka, Japan).

Statistics

The data from 3 independent experiments are expressed as the mean \pm standard deviation (SD). Three independent experiments were performed with cells derived from 3 different cultures. The data for comparison may be the same in some figures. It was used as a representative example because it has been reproduced more than once. We also took this step because it is extremely difficult to conduct all the experiments on the same day. Comparison between the 2 groups was performed using the student's *t*-test. One-way ANOVA with Dunnett's test was performed for multiple comparisons. $P < 0.05$ was considered significant.

Results

All of the Tested CRR Cell Lines Were Resistant to H₂O₂-Induced Cell Death

In order to determine the sensitivity of CRR cells to H₂O₂, WST assays were conducted (Figure 1). The WST assay results

showed that all of the CRR cell lines were significantly resistant to H₂O₂ compared to the corresponding parental cell lines. The H₂O₂ half maximal inhibitory concentration (IC₅₀) values for the HepG2, HepG2-8960-R, and HepG2-R cells were approximately 13, 25, and 45 μ M, respectively. The H₂O₂ IC₅₀ values for the SAS and SAS-R cells were approximately 20 and 30 μ M, respectively. Lastly, the H₂O₂ IC₅₀ values for the HeLa and HeLa-R cells were approximately 5 and 15 μ M, respectively.

H₂O₂-Resistant Cell Lines Were Not Resistant to X-Ray-Induced Cell Death

Several distinct cell types, such as murine lens, monkey kidney, and hamster ovarian cells, have been found to acquire H₂O₂ resistance after conditioning with H₂O₂.¹²⁻¹⁵ In order to determine whether H₂O₂-resistant cells were also resistant to X-rays, we generated H₂O₂-resistant cells from HepG2, SAS, and HeLa cells. As shown in Figure 2, we successfully obtained H₂O₂-resistant cells, which are referred to by their cell line name followed by H₂O₂ to indicate their H₂O₂ resistance. HepG2-H₂O₂, SAS-H₂O₂, and HeLa-H₂O₂ cells continued to proliferate in medium containing up to 50 μ M of H₂O₂, respectively. An MHDS assay revealed that the X-ray sensitivity of HepG2-H₂O₂ cells was nearly identical to that of parental

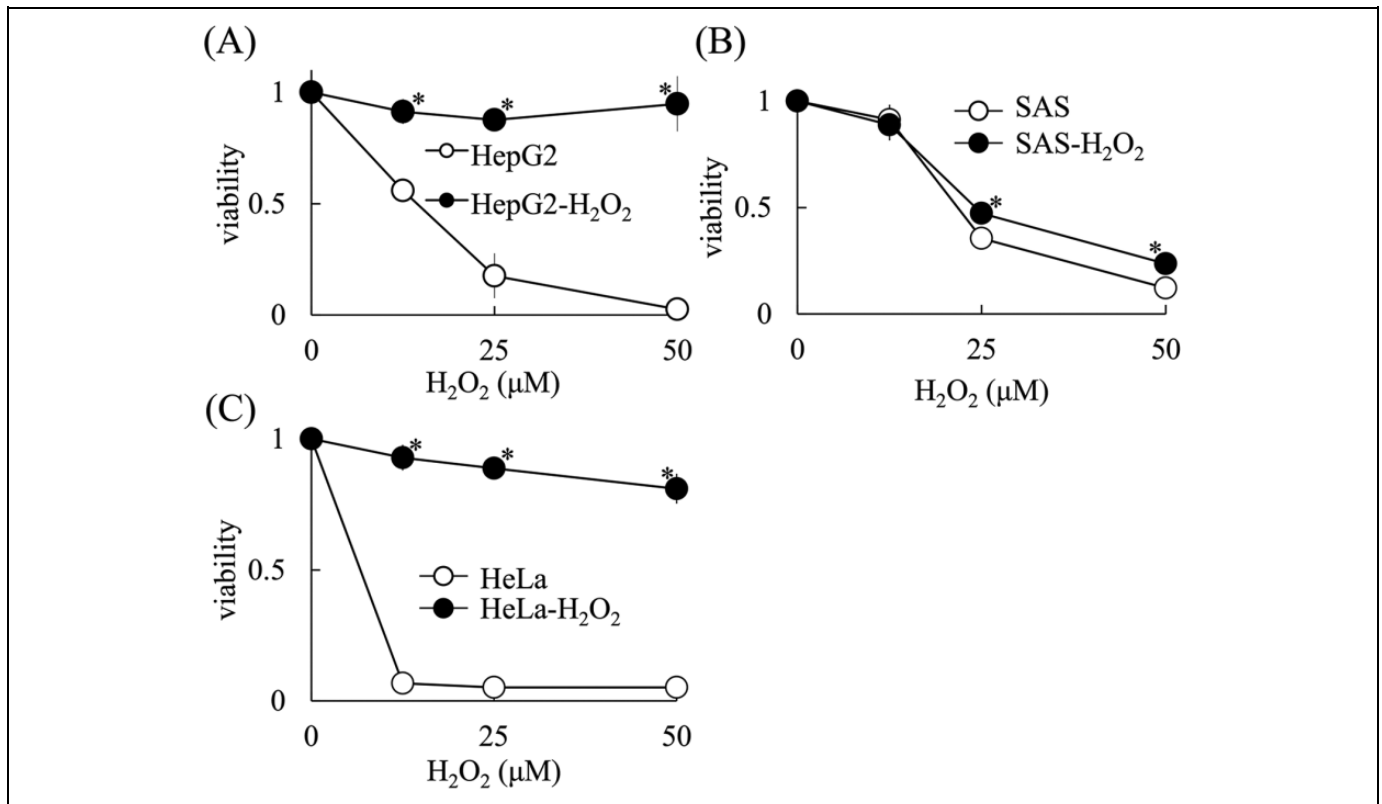


Figure 2. Generation of H₂O₂-resistant cell lines. The sensitivity of cells to H₂O₂-induced cell death was determined by WST assays. The H₂O₂-resistant cell lines are referred to as their cell line name followed by H₂O₂. The cells (5×10^3) were incubated with H₂O₂ for 48 hours and a WST assay was performed according to the manufacturer's protocol. A, H₂O₂ sensitivity of parental HepG2 and HepG2-H₂O₂ cells. B, H₂O₂ sensitivity of parental SAS and SAS-H₂O₂ cells. C, H₂O₂ sensitivity of parental HeLa and HeLa-H₂O₂ cells. The data represent the mean \pm SD of 3 independent experiments and are presented relative to the control parental cell lines. *: $p < 0.05$ using Student's t test (vs. Parental cells).

HepG2 cells (Figure 3). Additionally, Figure 3 shows that SAS-H₂O₂ and HeLa-H₂O₂ cells exhibited similar X-ray sensitivity as their parental SAS and HeLa cells, respectively.

Exposure to Fractionated X-Rays Induced H₂O₂ Resistance in Cancer Cells

HepG2 cells exposed to 0.5 Gy/day \times 10 of fractionated X-rays (referred to as HepG2-0.5 cells) acquired H₂O₂-resistant phenotype (Figure 4). The sensitivity of HepG2-0.5 cells to H₂O₂-induced cell death was nearly identical to that of the HepG2-8960-R cells. Further exposure of HepG2-0.5 cells to 1 Gy/day of fractionated X-rays (referred to as HepG2-0.5-1 cells) did not increase the H₂O₂ resistance. On the other hand, SAS cells did not acquire H₂O₂ resistance upon exposure to 0.5 Gy/day \times 10 of fractionated X-rays (referred to as SAS-0.5 cells). However, additional exposure of these cells to 1 Gy/day \times 10 of fractionated X-rays (referred to as SAS-0.5-1 cells) induced H₂O₂ resistance. In addition, the exposure of HeLa cells to 0.5 Gy/day \times 10 of fractionated X-rays (referred to as HeLa-0.5 cells) induced H₂O₂ resistance.

The H₂O₂ Resistance Phenotype of the CRR Cell Lines Was Irreversible

In order to maintain the X-ray resistance of CRR cells, they were exposed daily to 2 Gy of X-rays (maintenance irradiation, MI). In order to test whether H₂O₂ resistance in CRR cell lines is reversible, we cultured CRR cells without MI for 3 months, and then determined the H₂O₂ sensitivity of these cells by WST assays. The CRR cells cultured without MI for 3 months were still radioresistant (data not shown). WST assays indicated that the H₂O₂ sensitivity of HepG2-8960-R cells cultured without MI (referred to as HepG2-8960-R-NoIR cells) was nearly identical to that of HepG2-8960-R cells (Figure 5A). Furthermore, WST assays indicated that the H₂O₂ sensitivity of HepG2-R cells cultured without MI (referred to as HepG2-R-NoIR cells) was nearly identical to that of HepG2-R cells (Figure 5B). Moreover, WST assays indicated that the H₂O₂ sensitivity of SAS-R cells cultured without MI (referred to as SAS-R-NoIR cells) was nearly identical to that of SAS-R cells (Figure 5C). In contrast, WST assays showed that the H₂O₂ sensitivity of HeLa-R cells cultured without MI (referred to as HeLa-R-NoIR cells) was reduced compared to that of HeLa-R cells. Thus,

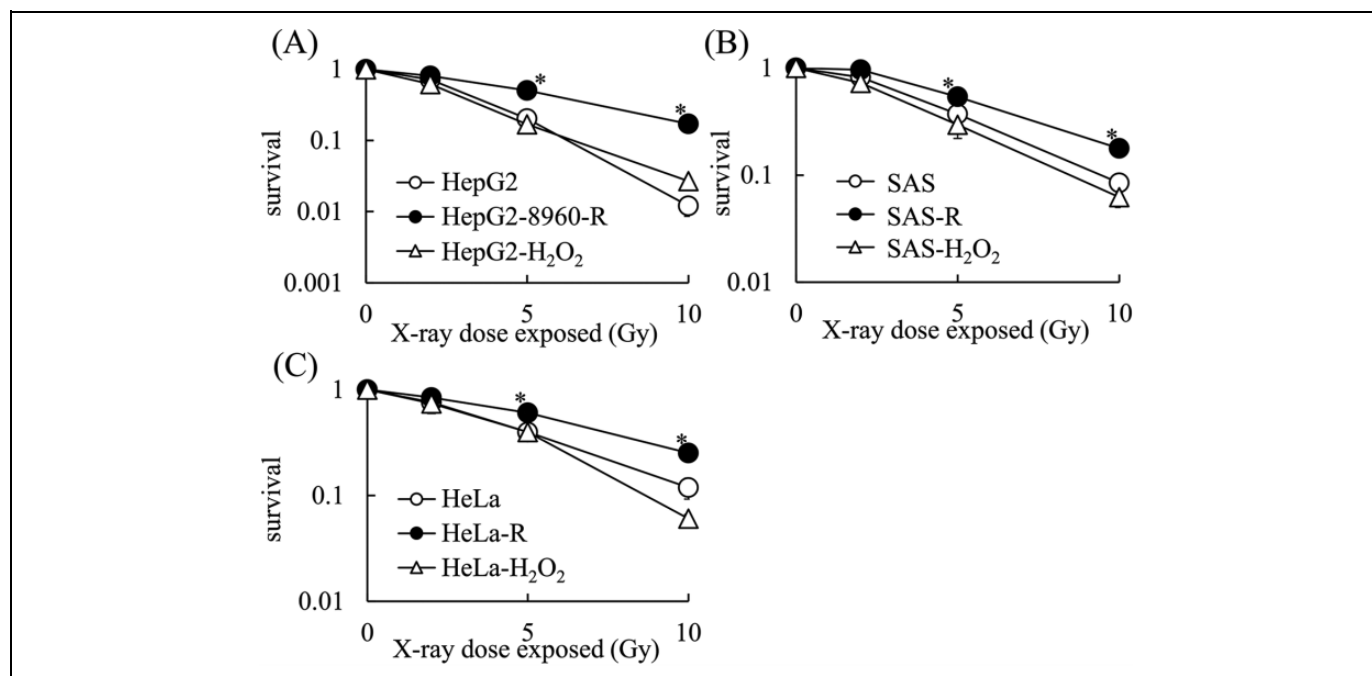


Figure 3. H₂O₂-resistant cell lines are not resistant to X-ray-induced cell death. A, X-ray sensitivity of HepG2, CRR HepG2-8960-R, and HepG2-H₂O₂ cells. B, X-ray sensitivity of SAS, CRR SAS-R, and SAS-H₂O₂ cells. C, X-ray sensitivity of HeLa, CRR HeLa-R, and HeLa-H₂O₂ cells. The data represent the mean ± SD of 3 independent experiments and are presented relative to the control parental cell lines. *: *p* < 0.05 using Dunnett’s test (vs. Parental cells).

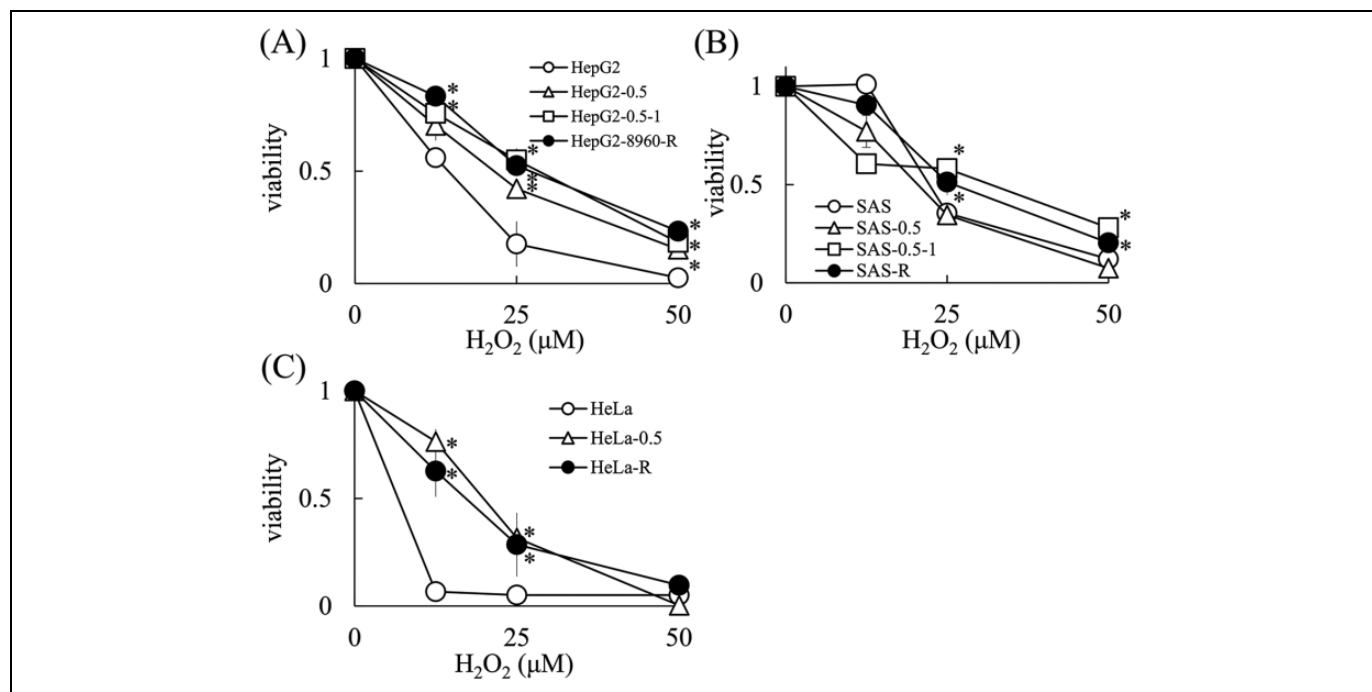


Figure 4. Exposure to fractionated X-rays induced H₂O₂ resistance in cancer cells. Cells (5×10^3) were incubated with H₂O₂ for 48 hours and a WST assay was performed according to the manufacturer’s protocol. A, H₂O₂ sensitivities of parental HepG2 cell, 0.5 Gy/day × 10 of fractionated X-rays pretreated HepG2 (HepG2-0.5) cell, 0.5 Gy/day × 10 plus 1 Gy/day × 10 of fractionated X-ray pretreated HepG2 (HepG2-0.5-1) cell and CRR HepG2-8960-R cell. B, H₂O₂ sensitivities of parental SAS, 0.5 Gy/day × 10 of fractionated X-rays pretreated SAS (SAS-0.5) cell, 0.5 Gy/day × 10 plus 1 Gy/day × 10 of fractionated X-ray pretreated SAS (SAS-0.5-1) cell and CRR SAS-R cells. C, H₂O₂ sensitivity of parental HeLa, 0.5 Gy/day × 10 of fractionated X-rays pretreated HeLa (HeLa-0.5) cell and CRR HeLa-R cell. The data represent the mean ± SD of 3 independent experiments and are presented relative to the control parental cell lines. *: *p* < 0.05 using Dunnett’s test (vs. Parental cells).

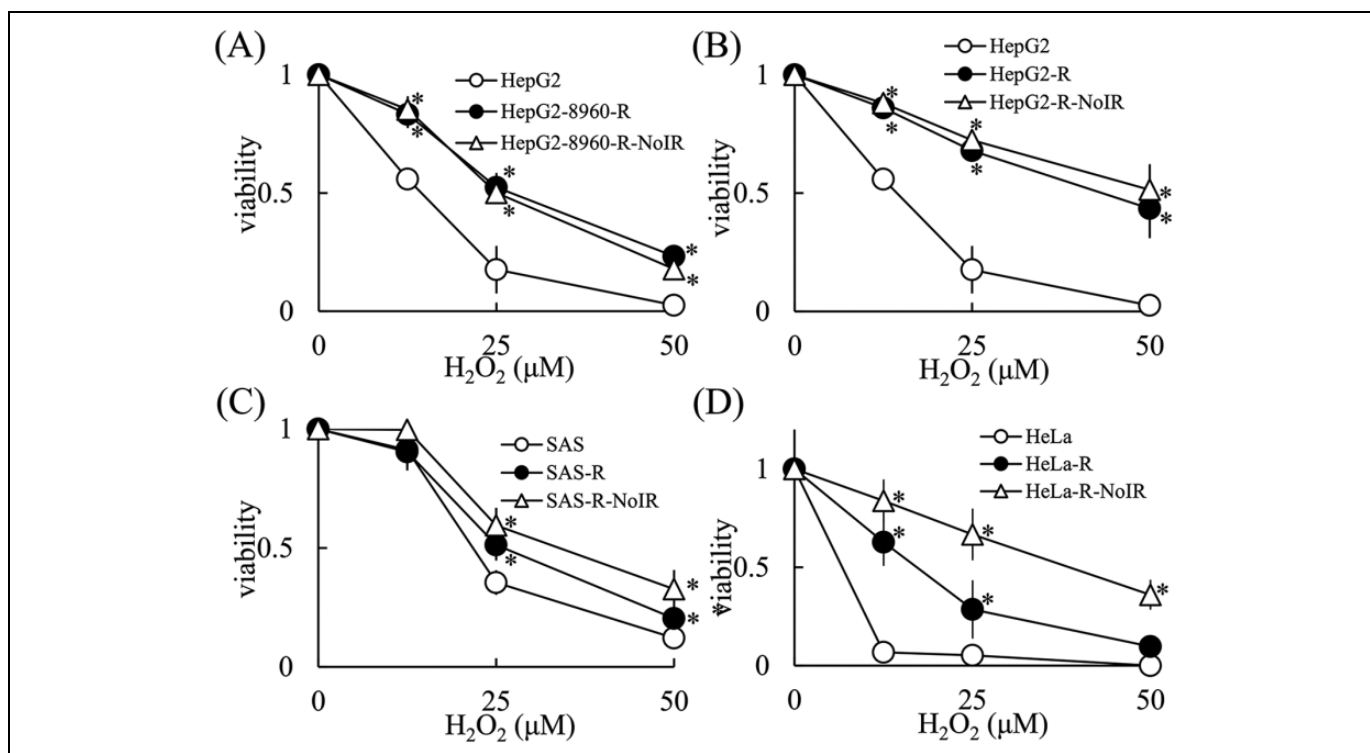


Figure 5. The H_2O_2 resistance phenotype of CRR cell lines was irreversible. Cells (5×10^3) were incubated with H_2O_2 for 48 hours and a WST assay was performed according to the manufacturer's protocol. A, H_2O_2 sensitivity of parental HepG2, CRR HepG2-8960-R, and HepG2-8960-R-NoIR cells. B, H_2O_2 sensitivity of parental HepG2, CRR HepG2-R, and HepG2-R-NoIR cells. C, H_2O_2 sensitivity of parental SAS, CRR SAS-R, and SAS-R-NoIR cells. D, H_2O_2 sensitivity of parental HeLa, CRR HeLa-R, and HeLa-R-NoIR cells. The data represent the mean \pm SD of 3 independent experiments and are presented relative to the control parental cell lines. *: $p < 0.05$ using Dunnett's test (vs. Parental cells).

HeLa-R-NoIR cells were more resistant to H_2O_2 compared with HeLa-R cells. These results suggested that H_2O_2 resistance in CRR cell lines was irreversible.

Exposure to X-Rays Induced H_2O_2 Resistance in CRR Cell Lines

The effects of H_2O_2 after X-ray exposure were evaluated utilizing WST assays. As expected, 10 Gy of X-rays sensitized parental HepG2, SAS, and HeLa cells to H_2O_2 (Figure 6). In contrast, X-rays induced further H_2O_2 resistance in all of the tested CRR cell lines including HepG2-8960-R, HepG2-R, SAS-R, and HeLa-R.

The Kinetics of Intracellular ROS Production in H_2O_2 -Treated Parental and CRR Cells

The intracellular ROS levels were quantitated using the CellROXTM Green Reagent. In untreated cells, the amount of intracellular ROS was higher in HepG2-8960-R cells compared to HepG2 cells (Figure 7). After treatment with 100 μM of H_2O_2 for 3 hours, the intracellular ROS level was increased in HepG2 cells compared to untreated cells. Additionally, the intracellular ROS level decreased in HepG2 cells after H_2O_2 treatment for 24 hours. In contrast, in HepG2-8960-R cells, a significant increase in intracellular ROS was not observed after H_2O_2

treatment for 3 or 24 hours. These tendencies were also observed in SAS, SAS-R, HeLa and HeLa-R.

Discussion

One of the main obstacles of radiation therapy for cancer treatment is the existence of radioresistant cells. In order to understand the characteristics of radioresistant cells, CRR cell lines have been generated. Screening for anticancer drugs against CRR cell lines elucidated that all CRR cells were resistant to docetaxel (an inhibitor of microtubule depolymerization) compared with their parental cells.¹⁶ Recently, Tomita et al. found that the CRR cell lines, SAS-R and HeLa-R, were resistant to H_2O_2 compared to their corresponding parental SAS and HeLa cell lines, respectively.⁷ They suggested that the membrane status is a major factor conferring H_2O_2 resistance in CRR cells. In this study, we generated CRR cell lines by exposing parental cell lines to a stepwise increase in fractionated X-rays from 0.5 to 2 Gy/day. We then examined whether our established CRR cell lines were resistant to H_2O_2 . We found that SAS-R, HeLa-R, HepG2-8960-R and HepG2-R cell lines were all resistant to H_2O_2 . We also found that the H_2O_2 -resistant phenotype is an irreversible characteristic of the CRR cell lines. Our results (Figure 4) indicated that CRR cells acquired H_2O_2 resistance during the development of radioresistance,

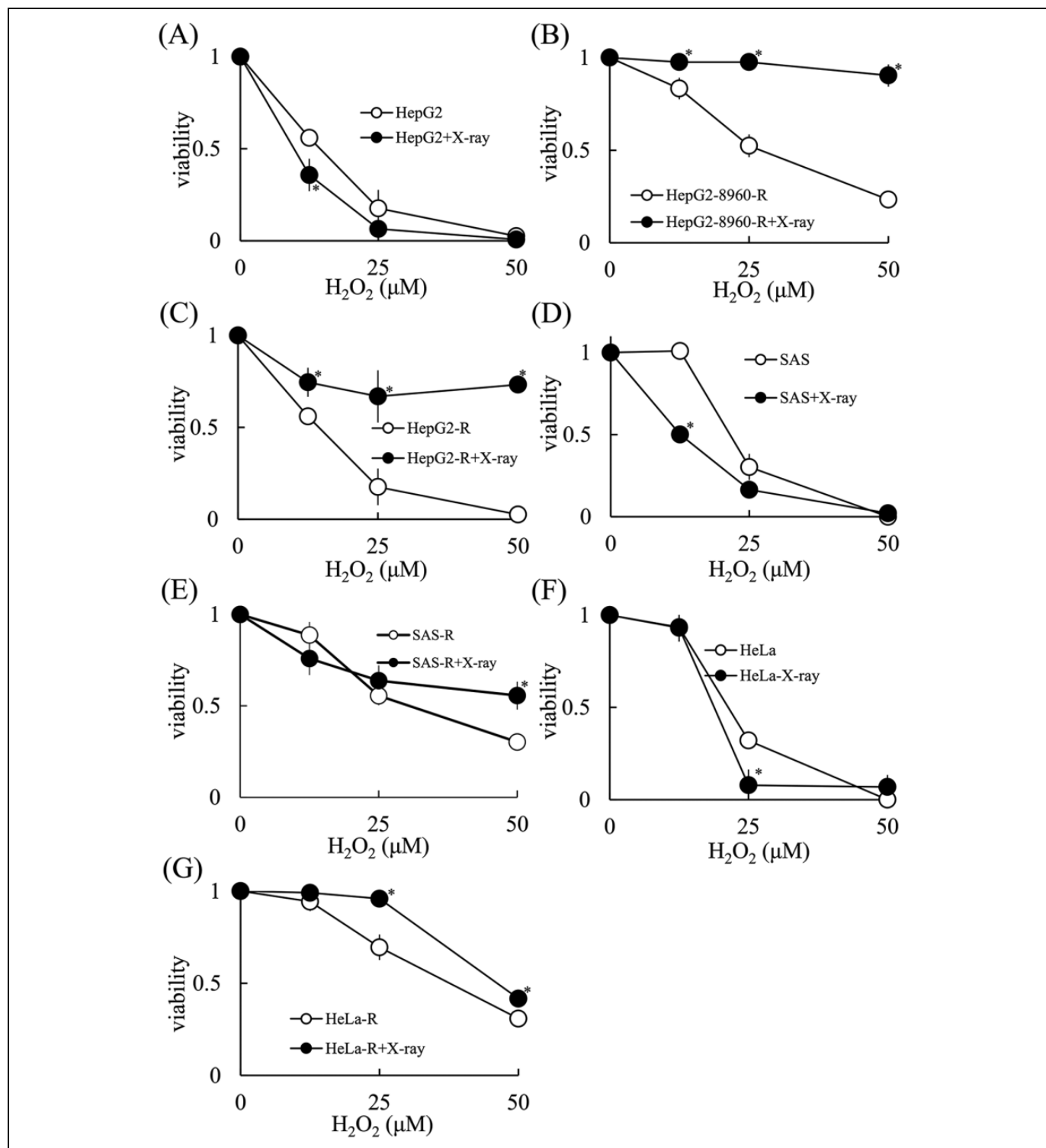


Figure 6. Exposure of CRR cells to X-rays enhanced their resistance to H₂O₂-mediated cell death. Following incubation with H₂O₂ for 1-h, cells (5×10^3) was irradiated with 10 Gy of X-rays and incubated extra 48 hours. WST assays were then performed according to the manufacturer's protocol. A, H₂O₂ sensitivity of parental HepG2 and HepG2 irradiated cells. B, H₂O₂ sensitivity of CRR HepG2-8960-R and CRR HepG2-8960-R irradiated cells. C, H₂O₂ sensitivity of HepG2-R and HepG2-R irradiated cells. D, H₂O₂ sensitivity of Parental SAS and SAS irradiated cells. E, H₂O₂ sensitivity of CRR SAS-R and SAS-R irradiated cells. F, H₂O₂ sensitivity of parental HeLa and HeLa irradiated cells. G, H₂O₂ sensitivity of CRR HeLa-R and HeLa-R irradiated cells. The data represent the mean \pm SD of 3 independent experiments and are presented relative to the control parental or CRR cell lines. *: $p < 0.05$ using Student's *t* test (vs. no irradiated cells).

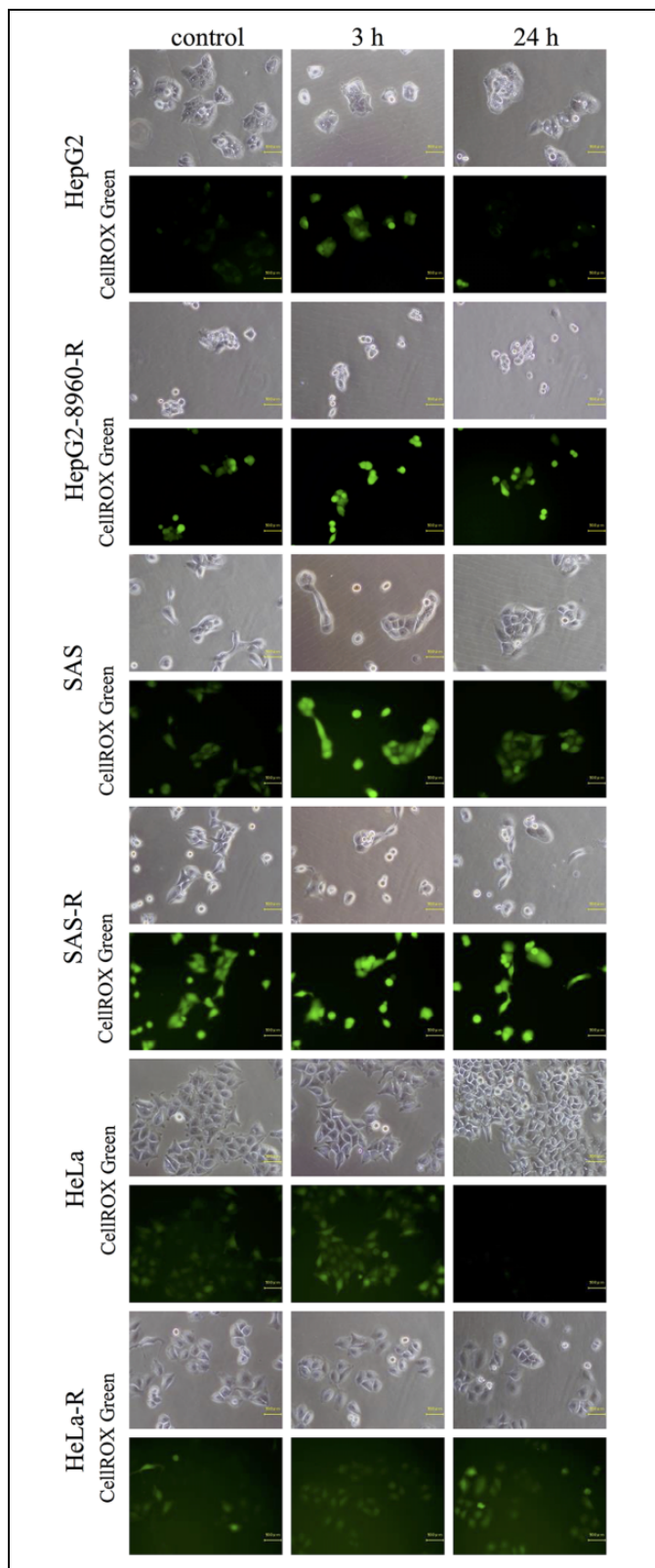


Figure 7. The kinetics of intracellular ROS production in H_2O_2 -treated parental and CRR cells. Cells were treated with or without $100 \mu\text{M}$ of H_2O_2 for 3 or 24 hours and the intracellular ROS levels were determined using the CelliROX™ Green Reagent according to the manufacturer's protocol.

suggesting that tumors subjected to radiotherapy might possess H_2O_2 resistance.

In this study, we developed H_2O_2 -resistant cell lines and evaluated their sensitivity to radiation. Unexpectedly, the H_2O_2 -resistant cells did not exhibit radioresistance compared to their parental cells (Figure 3). Exposure of cells to ionizing radiation leads to the formation of ROS (including H_2O_2), which is associated with radiation-induced cytotoxicity.^{17,18} Therefore, ROS scavengers are one of the important factors that protect cells against ROS-induced injury during ionizing radiation exposure. Previous studies showed that H_2O_2 -resistant cells had increased catalase expression.^{19,20} Catalase is a potent antioxidant enzyme that converts H_2O_2 into hydrogen and water. Tomita et al. reported that catalase activity was lower in CRR cells compared to parental cells, despite higher H_2O_2 resistance. Previous reports showed that H_2O_2 -induced DNA damage is distinct from ionizing radiation-induced DNA damage.²¹ H_2O_2 produces hydroxyl radical damage in the form of DNA single-strand breaks.²² DNA double-strand breaks (dsbs) induced by ionizing radiation are the most deleterious lesion that induces cell death. H_2O_2 does not produce DNA dsbs. It has been reported that there was an increase in γH2AX -positive cells in the parent cell line after irradiation, but not in CRR cells.²² From these results we hypothesize that CRR cells can efficiently repair not only DNA dsbs but also DNA single-strand breaks. Therefore, CRR cells are resistant to H_2O_2 .

Radiotherapy is one of the most common treatments for cancer. However, the existence or induction of radioresistant cells is one of the major problems to overcome in this therapy. Therefore, approaches utilizing a sensitizer in combination with radiation therapy have been proposed, and many drugs have been tested for this purpose. As we previously reported, Everolimus is one candidate.²³ H_2O_2 is another candidate and KORTUC therapy is currently being used to treat multiple cancer types.²⁴⁻²⁶ In biological systems, H_2O_2 is converted into a more reactive species (e.g. hydroxyl radical) via the Fenton reaction, which is a transition metal-catalyzed reaction.²⁷ These reactive species injure vital molecules including DNA, proteins, and membrane lipids, which leads to the destruction of cellular integrity. Several enzymes scavenge these toxic oxidants or transform them into nontoxic metabolites; e.g. catalase decomposes H_2O_2 to water and molecular oxygen. Since hydroxyl radicals are a direct product of H_2O_2 catalysis, H_2O_2 may generate greater amounts of hydroxyl radicals compared to radiation therapy. Ogawa theorized that when tumors are irradiated in the presence of H_2O_2 , the activities of antioxidative enzymes such as peroxidase and catalase are blocked, and oxygen molecules are produced at the same time via the H_2O_2 effect. This results in oxidative damage to low-LET radioresistant tumor cells, thereby rendering them highly sensitive to irradiation.⁸ It should be noted that the overall levels of intracellular ROS in CRR cells were abundant compared to the levels in parental cells (Figure 7). Treatment with H_2O_2 increased intracellular ROS in HepG2 cells but not in HepG2-8960-R cells (Figure 7). We hypothesize that

antioxidative enzymes are blocked in parental cells, which renders them highly sensitive to radiation. Due to the overall high amount of intracellular ROS in CRR cells, treatment with H₂O₂ might have no effect on CRR cells. Tomita et al. reported that catalase activity was lower in CRR cells compared to parental cells.²⁸ High levels of intracellular ROS may inactivate catalase in CRR cells. We hypothesize that internal ROS scavenging activity, such as via catalase activity, may not be a major factor contributing to H₂O₂ resistance in CRR cells. In CRR cells, it has been reported that the amount of Fe²⁺ was lower than that in the parental cells.²⁹ Furthermore, regarding the amount of hydroxyl radicals, it has been reported that the amount of hydroxyl radicals in CRR cells is lower than that in the parental cells.^{28,29} These results indicate that the Fenton reaction is one of the key factor in the resistant phenotype in CRR cells because it generates hydroxyl radicals and oxidize the plasma membrane finally leads to cell death.

In this study, we found that fractionated X-ray exposure induced H₂O₂ resistance in CRR cells. Therefore, it is necessary to carry out cancer therapy such as KORTUC with the presence of these resistant cells in mind, and as the next stage, it would be necessary to investigate the appearance rate of these cells immediately and take countermeasures.

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Authors' Note

This study does not include animal studies or human studies that require ethical review. The origin of the cell lines used in this study was stated in materials and methods section.


Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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