Radiation-induced Apoptosis, Necrosis and G2 Arrest in Fadu and Hep2 Cells

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ABSTRACT

Purpose : Radiation damage is produced and viable cell number is reduced. We need to know the type of cell death by the ionizing radiation and the amount and duration of cell cycle arrest. In this study, we want to identified the main cause of the cellular damage in the oral cancer cells and normal keratinocytes with clinically useful radiation dosage.

Materials and Methods : Human gingival tissue specimens obtained from healthy volunteers were used for primary culture of the normal human oral keratinocytes (NHOK). Primary NHOK were prepared from separated epithelial tissue and maintained in keratinocyte growth medium containing 0.15 mM calcium and a supplementary growth factor bullet kit. Fadu and Hep-2 cell lines were obtained from KCLB. Cells were irradiated in a ^{137}Cs γ-irradiator at the dose of 10 Gy. The dose rate was 5.38 Gy/min. The necrotic cell death was examined with Lactate Dehydrogenase (LDH) activity in the culture medium. Every 4 day after irradiation, LDH activities were read and compared control group. Cell cycle phase distribution and preG1-incidence after radiation were analyzed by flow cytometry using Propidium Iodine staining. Cell cycle analysis were carried out with a FAC Star plus flowcytometry (FACS, Becton Dickinson, USA) and DNA histograms were processed with CELLFIT software (Becton Dickinson, USA).

Results : LDH activity increased in all of the experimental cells by the times. This pattern could be seen in the non-irradiated cells, and there was no difference between the non-irradiated cells and irradiated cells. We detected an induction of apoptosis after irradiation with a single dose of 10 Gy. The maximal rate of apoptosis ranged from 4.0% to 8.0% 4 days after irradiation. In all experimental cells, we detected G2/M arrest after irradiation with a single dose of 10 Gy. Yet there were differences in the number of G2/M arrested cells. The maximal rate of the G2/M ranges from 60.0% to 80.0% 24h after irradiation. There is no significant changes on the rate of the G0/G1 phase.

Conclusion : Radiation sensitivity was not related with necrosis but cell cycle arrest and apoptosis. These data suggested that more arrested cell is correlated with more apoptosis. ***(Korean J Oral Maxillofac Radiol 2000 ; 30 : 275-279)***

KEY WORDS : irradiation, Apoptosis, Necrosis, Cell Cycle

Introduction

When cells are irradiated, damage is produced and viable cell number is reduced. Recent reports suggest that the radiation induce apoptotic cell death with 1-10 Gy.[1-3] Apoptotic cell death can be distinguished from necrotic cell death.[4] Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptotic cell death is characterized by controlled autodigestion of the cell. Cells appear to initiated their own apoptotic death through the activation of endogenous proteases.[5,6] This results in cytoskeletal disruption, cell shrinkage, and membrane blebbing. In contrast, necrotic cell death is associated with an early loss of cell membrane integrity, resulting in leakage of cytoplasmic contents and the induction of an inflammatory response. Radiation induce cell death by causing damage that induces the cell to commit suicide. Some reports showed the radiation effect was different by the dose showing the result that Molt-4 cells at doses > 100 Gy and L5178Y cells at doses > 300 Gy showed typical necrosis, however, cell death in Molt-4 cells after 2-30 Gy had features of both apoptosis and necrosis.[7] Other reports suggested that radiation damage induced by varies doses of radiation as a function of time after exposure was

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only quantitatively but not qualitatively different.[8] However, irradiated cultures of cells show a wide variety of morphological and biological abnormalities. These included nuclear fragmentation and other evidence of programmed cell death, but they also included a considerable amount of lysis, necrosis, and persistent abnormal growth and function, which were expressed in the progeny of irradiated cells.[9] The dose dependence of these various responses is documented, because it probably determines to a large extent the outcome of radiation exposure in terms of whether a cell dies, divides normally, or develops genomic instability, mutation, and ultimate carcinogenic progression of the progeny.[9] In clinical useful dosage, apoptosis was believed to play very important role. Treatments that restore the ability to properly regulate apoptosis could also be of considerable benefit in some malignancies. Several observations suggest that the central mediators of apoptosis may be pharmacologically manipulated in a cell specific fashion.[10] Individual tissues in the body can vary significantly in the expression of individual members on the apoptotic related gene families. If that is above, does the variety of the radiosensitivity of human cells and tumors depend on the amount of apoptotic cell death? Cell cycle control has widely been accepted to play an important role in the response of cells to DNA-damaging agents.[11] Cells can arrest in the G1 or G2 phases of the cell cycle following DNA damage caused by ionizing radiation in attempts to repair or recover from the induction of DNA lesions.[12] However, much of the arrested cells go down the death.[13] We need to know the type of cell death by the ionizing radiation and the amount and duration of cell cycle arrest. In this study, we want to identified the main cause of the cellular damage in the oral cancer cells and normal keratinocytes with clinical useful radiation dosage.

Materials and Methods

1. Cell Lines and Culture Condition

Culture of primary NHOK. Human gingival tissue specimens are obtained from healthy volunteers undergoing oral surgery. The tissue samples were thoroughly established three times with calcium-and magnesium-free Hank's balanced salt solution (CMF-HBSS;Gibco/BRL, Grand Island, NY). To separate the epithelium from the underlying submucosa, the tissues were incubated in CMF-HBSS containing collagenase (type II, 1.0 mg/ml; Sigma Chemical Co., St. Louis, MO) and dispase (grade II, 2.4 mg/ml; Boehringer-Mannheim, Indianapolis, IN) for 90 min at 37cc in 95% air and 5% CO_2. Primary NHOK were prepared from separated epithelial tissue and maintained in keratinocyte growth medium (KGM; Clonetics Corp., Sam Diego, CA) containg 0.15 mM calcium and a supplementary growth factor bullet kit. Approximately 70% confluent primary NHOK were plated at 1×10^5 cells per 60-mm petri dish, cultured for 7 days, and then subcultured once to use for experiments.

Culture of cancer cell lines. Fadu and HEp-2 cell lines were obtained from KCLB (Seoul, Korea). They were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY.), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY), 100 U/ml penicillin, 100 mg/ml streptomycin. All the cells of two lines were grown in the dark at 37°C in a 5% CO_2 atmosphere.

2. Irradiation

Cells were irradiated in a ^{137}Cs γ-irradiator (IBL 437C, France) at the dose of 10 Gy. The dose rate was 5.38 Gy/min. Cultures were always irradiated on ice to minimize the amount of repair occurring during the irradiation period.

3. Assaying of necrosis

The necrotic cell death was examined with Lactate Dehydrogenase (LDH) activity in the culture medium. The experimental cells were seeded at 1×10^5 cells with 5 ml of the culture medium onto 35 mm dish, and then incubation and irradiation as scheduled. Every 4 day after irradiation, LDH activities were read and compared control group. During the culture period, we did not change the media. Aliquots (50 μl) of the resultant media were transferred from all wells to the fresh 96 well flat-bottom plate and used for LDH assaying. As negetive controls, culture media were collected at 0 time incubation and used to determine the background release of LDH. An assay kit for LDH (Cytotoxicity 96 assay for LDH, Promega, USA) was used for determination of LDH activity according to the manufacturer's protocol based on NAD-dependent catalysis of tetrazolium salt into red formazan product. The results were expressed as a optical density of the formazan. Absorbance was recorded at 490 mn with ELISA reader (Bio-tech Instruments Inc., Model EL308, USA) within one hour after the addition of Stop Solution.

4. Flow cytometric analysis

Cell cycle phase distribution and preG1-incidence after radiation was analyzed by flow cytometry using Propidium Iodine (PI) (Sigma, Germany) staining. Briefly, control and

treated cells were harvested by trypsinization, then fixed in 70% ethanol and stored at 4°C for up to 7 days before DNA analysis. After the removal of ethanol by centrifugation.(1800 rmp, 4 min), the fixed cells washed twice with 0.1% bovine serum albumin (BSA) (Amresco, USA) containing 5 ml phosphate buffered saline (PBS) (GibcoBRL, USA). Each tube was added 25 μl PI, 25 μl RNase (Sigma, Germany) up to 200 μl PBS at 37°C for 30 min. Before flow cytometric analysis, samples were protected from light. Cell cycle analysis were carried out with a FAC Star plus flowcytometry (FACS, Becton Dickinson, USA) and DNA histograms were processed with CELLFIT software (Becton Dickinson, USA).

Results

1. Ionizing radiation could not induce necrosis

LDH activity increased in all of the experimental cells by the times. This pattern could be seen in the non-irradiated cells, and there was no difference between the non-irradiated cells and irradiated cells (Fig. 1).

2. Pre G1 peak increased by radiation

In all experimental cells, we detected an induction of apoptosis after irradiation with a single dose of 10 Gy (Fig. 2). The maximal rate of apoptosis ranged from 4.0% to 8.0% 4 days after irradiation.

3. Cells arrested in the G2 phase of the cell cycle by ionizing radiation

In all experimental cells, we detected G2/M arrest after irradiation with a single dose of 10 Gy. Yet there were differences in the number of G2/M arrested cells. The maximal rate of the G2/M ranged from 60.0% to 80.0% 1 day after irradiation. There was no significant changes on the rate of the G0/G1 phase (Fig. 3).

Discussion

In all of the cell lines, we found a G2/M arrest and an induction of apoptosis, but the maximal rate of apoptosis with a range from 4.0 to 8.0% was rather small. In vitro study, apoptotic dead cells are to be floated not to be regurgitated by macrophages. Our results were the spot rates of the apoptotic cells and for the calculating of the total apoptotic cells, the apoptosis rates should be summated. There are many studies about the radiation sensitivity and the effect of several agents on changing the radiation sensitivity of the tumor cells.[14, 15] However they have reported about only tumor and there was no comparison with normal cells. In our study we compared the radiation response of two human oral cancer cell lines with NHOK. The rates of apoptosis and G2/M arrest after

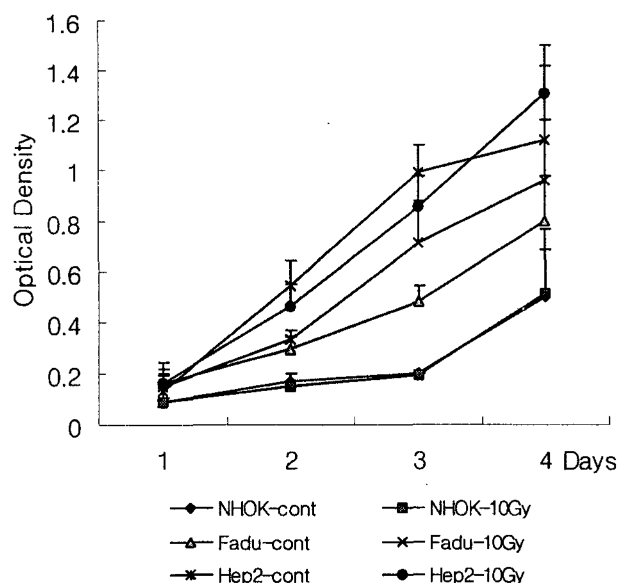

Fig. 1. LDH in supernatant (n = 3).

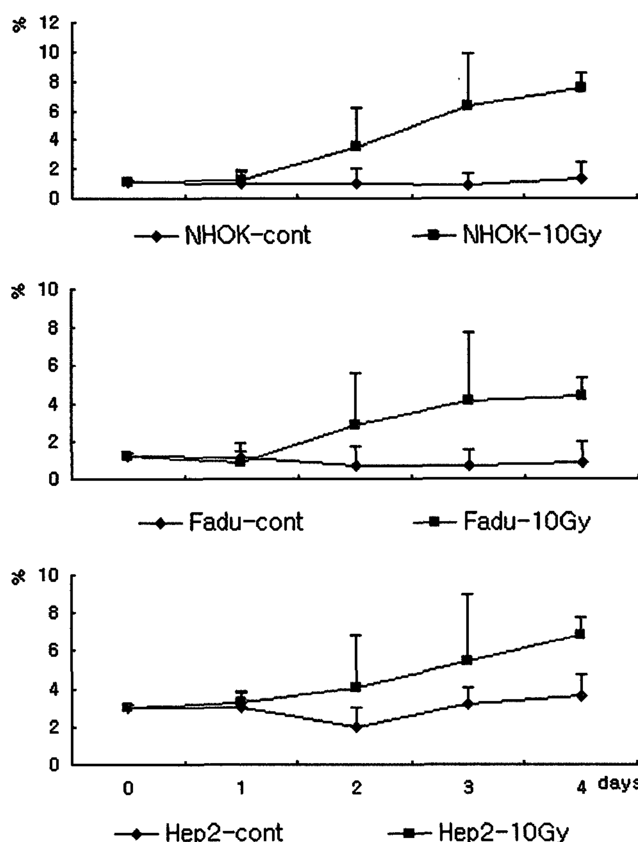

Fig. 2. Rates of the pre-G1 peak in cell cycle after irradiation (n = 3).

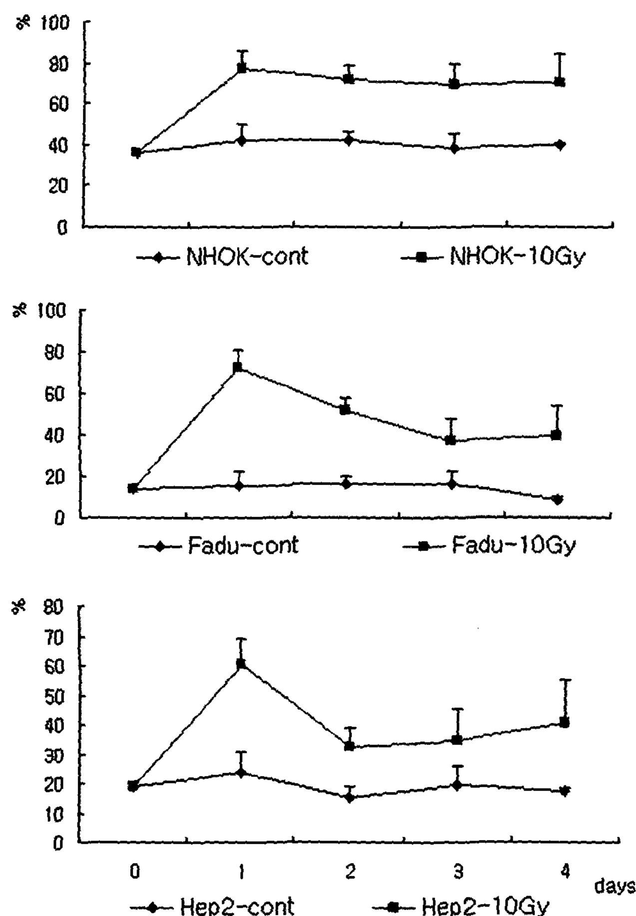

Fig. 3. Rates of the G2 areas in cell cycle after irradiation (n = 3).

irradiation were largest in the NHOK. Some reports showed that the decrease of the number of G2/M cells correlated with the induction of apoptosis. For instance, a decrease in the irradiation-induced G2/M arrest is correlated with a higher rate of apoptosis.[16,17] Tamamoto et al.[18] have recently reported that radioresistance of human cancer cells is closely correlated with r-ray-induced G2 arrest because that cellular machinery for avoiding cell death appears to be generated during G2 arrest. However, both of the G2/M arrest and apoptosis were seen the highest rates in NHOK, this was the different result compared with previous studies. These data suggest that a increase in the irradiation-induced G2/M arrest is not correlated with a lower rate of apoptosis when we compare it in several kinds of cells. Apoptosis would be induced rather more in the cells presenting massive G2/M arrest. G2/M arrest From our present results, it is considered that arrested cells might go down apoptosis. In facts, many of the genes regulating the G2 arrest are related to the apoptosis.[19-21] On cell cycle checkpoints, some protein can influence the response to DNA damage by channelling cells into an apoptotic pathway.

LDH assays were usually used to quantify cell damage. LDH, a component of the cell, is a stable cytoplasmic enzyme that is released on cell lysis, necrosis.[22] When the amount of LDH in the culture medium was measured four days after irradiation, the optical density signifying the LDH density, showed a tendency to increase. However, there was no significant difference in the supernatant between control and irradiated cells. It means that the necrosis had no responsibility for radiation damage and diversity of the radiation sensitivity.

The effect of radiation on NHOK and tumor cell lines has been examined. Results indicated that radiation sensitivity was related with not necrosis but cell cycle arrest and apoptosis. These data suggested that more arrested cell is correlated with more apoptosis. So, in further studies, we should work to reveal the genes related both of the G2 cell arrest and apoptosis.

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