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p53 Nuclear Accumulation as a Possible Biomarker for Biological Radio-dosimetry in Oral Mucosal Epithelial Cells

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Cellular response to ionizing radiation is affected by cell types, radiation doses, and post-irradiation time. Based on the trypan blue dye exclusion assay in normal oral mucosal cells (OM cells), a 48 h post-irradiation was sufficient and an adequate time point for the evaluation of radiation sensitivity. Its LD_{50} was approximately 1.83 Gy. To investigate possible biomarkers useful for the biological radiodosimetry of normal epithelial cells (p53, c-fos, cyclin D1, cdc-2, pRb) EGF receptor phosphorylation and Erk activation were evaluated at different radiation doses and different post-irradiation times. From 0.5 Gy, p53 was accumulated in the nucleus of basal cells of the OM raft culture at 4 h post-irradiation and sustained up to 24 h post-irradiation, which suggests that radiation-induced apoptosis or damage repair was not yet completed. The number of p53 positive cells and biosynthesis of p53 were correlated with radiation doses. Both cyclin D1 and c-fos were only transiently induced within 1 h post-irradiation. Cyclin D1 was induced at all radiation doses. However, c-fos induction was highest at 0.1 Gy, approximately 7.3 fold more induction than the control, whose induction was reduced in a reverse correlation with radiation dose. The phosphorylation pattern of cdc-2 and pRb were unaffected by radiation. In contrast to A431 cells overexpressing the EGF receptor approximately 8.5 fold higher than normal epithelial, the OM cells reduced the basal level of the EGF receptor phosphorylation in a radiation dose dependent fashion. In conclusion, among radiation-induced biomolecules, the p53 nuclear accumulation may be considered for the future development of a useful marker for biological radio-dosimetry in normal epithelial tissue since it was sustained for a longer period and showed a dose response relationship. Specific c-fos induction at a low dose may also be an important finding in this study. It needs to be studied further for the elucidation of its possible connection with the low dose radio-adaptive response.

Keywords: c-fos, EGF receptor phosphorylation, Oral mucosal epithelial cells, p53, γ-ray radiation

Introduction

Ionizing radiation provokes a variety of cellular and DNA damages. Many cellular proteins, such as cell cycle regulatory proteins (Fornace *et al.*, 1988), proto-oncogene transcription factors (Hollander *et al.*, 1989; Sheman *et al.*, 1989), and signal transducing molecules (Verheij *et al.*, 1996), are known to be involved in radiation-induced signal transduction leading to cell death, apoptosis, cellular repair of DNA damage, and cell survival. However, the radiation-induced response seems to be quite dependent on cell types and cellular status (Haimovitz-Friedman 1998). Again the cellular response to ionizing radiation varies according to radiation doses and post-irradiation time. For example, cancer cells such as A431 cells (high EGF receptor expressing cells) activates the EGF receptor within a short time after exposure to ionizing radiation (Schmidt-Ullrich *et al.*, 1997), but normal cells do not. Therefore the cellular status, radiation dose, and postradiation time may play important parameters for the estimation of the cellular radiation response at a given time.

The introduction of DNA damage to higher eukaryotic cells initiates many events. This includes the increased activity of p53, which subsequently activates p21/Waf (El-Diery *et al.*, 1993), and mdm2 (Perry *et al.*, 1993) and results in either cell cycle arrest or apoptosis (Kastan *et al.*, 1992). The induction of apoptosis by p53 is thought to play a critical role in the elimination of cells following DNA damage (Lowe *et al.*, 1993). Recently it was reported that p53 is induced upon g-ray radiation (Beham *et al.*, 1997).

C-jun and c-fos are proto-oncogenes whose products are

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members of the AP-1 (activator protein-1) family of transcription factors (Bohmann *et al.*, 1987) that play important roles in regulating proliferation, differentiation (McCabe *et al.*, 1995), transformation (Curran and Teich, 1982), and death (Smeyne *et al.*, 1993). Several groups have also examined the induction of proto-oncogenes following exposure to DNA-damaging agents or radiation. It has been demonstrated that the expression of the c-jun and c-fos genes is induced within 2 h after radiation in many cell types (Martin *et al.*, 1993; Collart *et al.*, 1995).

Several reports demonstrated that ionizing radiation can activate multiple signaling pathways (Verheij *et al.*, 1996). Radiation exposure, via activation of the epidermal growth factor (EGF) receptor, was shown to generate inositol triphosphate in order to induce Ca^{2+} oscillations, and to activate the mitogen activated protein (MAP) kinase pathway to a level similar to that observed by physiologic (~0.3 nM) EGF treatments (Schmidt-Ullrich *et al.*, 1997). However, it is unclear whether or not those effects on the EGF receptor may lead to a post-irradiation cell proliferation, or a low dose radiation-induced adaptive response.

In order to investigate which molecules shows the best dose-responsiveness at different post-irradiation times, we explored radiation-induced cellular responses based on radiation doses and post-irradiation times in normal primary cultured OM cells that are isolated from human gingival tissue or its raft culture.

Materials and Methods

Cell culture The normal oral mucosa (OM) cells were grown in the presence of Swiss 3T3 murine fibroblasts treated with mitomycin C (Kopan *et al.*, 1987), and were maintained with a growth medium consisting of Dulbeco's Modified Eagles Medium (DMEM; Gibco BRL, Grand, USA) and Ham's nutrient mixture F12 (Gibco BRL, Grand, USA) at a 3 : 1 ratio supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortison, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronin. The culture media were changed every two days, or at indicated times. After radiation, survival cells was counted based on dye exclusion after 0.4% trypan blue staining.

Radiation of cells OM cells were exposed to ^{137}Cs γ-ray (Atomic Energy of Canada Ltd., Canada) at a dose rate of 3.81 cGy/min.

Raft culture of human epidermal cells and immunohistochemistry of p53 Raft culture was followed as described previously with a few modifications (Son *et al.*, 1996). To prepare a dermal equivalent, Swiss 3T3 murine fibroblasts (3×10^5 cells/ml) were mixed with a type I collagen matrix reconstituted according to the manufacturer's specification (Nitta gelatin, Tokyo, Japan). Then, 2 ml of this mixture was plated onto a 30 mm filter chamber (3.0 μm Millicell-pc; Millipore Co., Bedford, USA.). OM cells were seeded at a density of 5×10^5 cells/30 mm Millicell on the dermal equivalent and cultured in a submerged state for 5 days and in an air-liquid interface state for two weeks. Cultures were maintained with a growth medium consisting of DMEM and Ham's nutrient mixture F12 at a 3 : 1 ratio supplemented with 10% fetal bovine serum (Hyclone, Logan, USA., FBS), 1×10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 2×10^{-11} M triiodothyronin. The culture media were changed every two days. At the end of the growth period, 0, 0.5, 2.0 and 8.0 Gy γ-ray were irradiated on the raft culture respectively. Then raft cultures were harvested at 4 h or 24 h after γ-ray radiation and fixed in Carnoy's solution (6 : 3 : 1; ethanol : chloroform : acetic acid) for 30 min. Fixed samples were embedded in paraffin, and sectioned (4 μm). For p53 staining, sections were heat-treated in a Tuf solution (Kreatech Biotechnology, Amsterdam Netherlands) for 5 min at 90°C before staining. A monoclonal antibody against p53 (DO7, Novocastra, Newcastle, UK) was used and were counterstained with Mayer's hematoxylin.

Biosynthetic labeling with [^{35}S] methionine and immunoprecipitation of p53, cyclin D1, c-jun, c-fos, cdc2, and Rb proteins Confluent OM cells in a submerged state were continuously labeled with 100 μCi/ml [^{35}S] methionine in a methionine free media for 1 h or 4 h post-irradiation. OM cells, were washed three times with PBS, extracted with a immunoprecipitation buffer (I. P. buffer: 1% Tx-100, 0.2% SDS, 0.15 M NaCl, 0.5% Soduimdeoxycholate, 50 mM Tris) supplemented with 2 mM PMSF. Insoluble proteins were removed by centrifugation at 12,000 rpm for 20 min. Protein concentrations of the supernatants were measured by a bicinchoninic acid protein assay. The protein was allowed to bind to a monoclonal antibody to human p53 (DO7, Novocastra, Newcastle, UK), a polyclonal antibody to human cyclin D1 (Rockland), a polyclonal antibody to human c-jun (Santa Cruz Biotechnology) and a monoclonal antibody to human c-fos (Santa Cruz Biotechnology) while rotating at 4°C. It was subsequently bound to protein G-sepharose 4B, washed three times with a I. P. buffer, once with a high salt buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4) and once with low salt buffer (10 mM Tris-HCl, pH 7.4). Polypeptides were analyzed by SDS-PAGE. For fluorography, the gel was soaked in 2 M sodium salicylate for 30 min, dried, and exposed to X-OMAT-AR film (Eastman Kodak) at -70°C.

Results

Cell survival curve of OM cells in monolayer culture after γ-radiation Cellular sensitivity to ionizing radiation varies between different cell types. To determine the radiation sensitivity of OM cells, primary cultured OM cells were plated at 20% confluency, irradiated at doses of 0.1, 0.5, 1.0, 2.0, 4.0, and 8.0 Gy and the cell survival was measured based on dye exclusion after tryphan blue staining (Fig. 1). The cell number increment of the non-irradiated plate (0.0 Gy) showed that the cell division cycle is between 24 h and 48 h. At 24 h post-irradiation, radiation-induced cell death was not prominent at a radiation dose lower than 8.0 Gy. However, at 8.0 Gy, cell death was prominent. This is probably due to radiation-induced cell death or necrosis. At 48 h post-irradiation when one round of cell division was completed after radiation, and radiation-induced apoptosis also has progressed, the radiation-dose dependent cell death was most

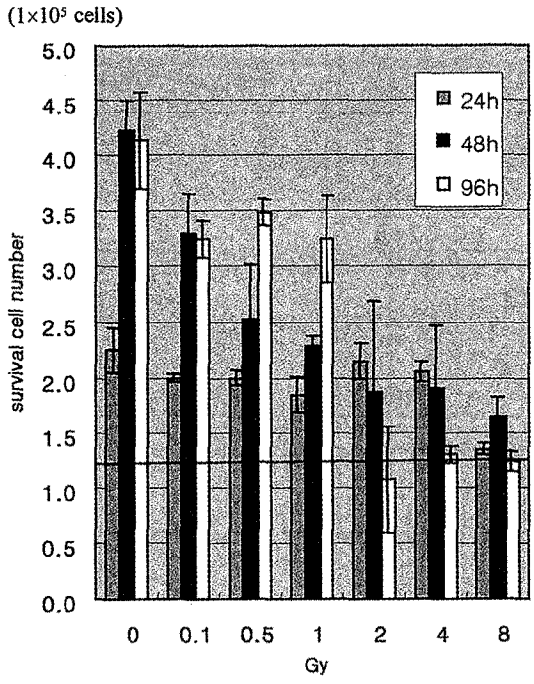

Fig. 1. Counting of survival cells by dye exclusion of the trypan blue staining in the irradiated OM cells. Cells were irradiated with ^{137}Cs γ-ray source of 3.81 Gy/min dose rate and were counted with trypan blue staining at 24 h, 48 h or 96 h post-irradiation time. Data represent mean value +/- S.D. of the three experiments.

prominently contrasted. At a 2.0 Gy radiation dose, the survival cells at 96 h post-irradiation further decreased more than the cell numbers at 48 h post-irradiation. This suggests that the radiation-induced apoptosis is going on until 96 h post-irradiation. At a radiation dose lower than 1.0 Gy, the repair process seems to be completed within a 48 h post-irradiation. Therefore, a 48 h post-irradiation may be a sufficient and adequate time period for the evaluation of the radiation sensitivity of OM cells. LD_{50} of OM cells at 48 h post-irradiation, which was determined from a calculation by a dose-effect analysis with microcomputers (Bisoft, Cambridge, UK), was approximately 1.83 Gy.

Accumulation of p53 in the basal cell layer of the OM raft culture and its disappearance are dependent on initial radiation doses as well as the post-irradiation time To investigate whether γ-ray radiation also affects p53 accumulation in the nucleus or not, and furthermore to explore its accumulation and disappearance time kinetics, the OM cells were 3-D raft-cultured for 14 days and were γ-irradiated on the outermost surface of the raft culture. The irradiated raft cultures were harvested at 4 h and 24 h post-irradiation time respectively, and the paraffin sections were immunohistochemically stained with the p53 monoclonal antibody (Fig. 2). In the control raft culture, basal cells were all negative in the p53 staining. However, upon γ-radiation at a dose of 0.5 Gy, the p53 protein apparently accumulated in the nucleus of the basal cells at 4 h post-irradiation. At 24 h post-irradiation, the p53 accumulation in the basal cell layer became weaker, but weak p53 positive cells were still detected at the 0.5 Gy radiation. This suggests that a 24 h post-irradiation may not be sufficient time for the repair of radiation-induced DNA damage at this radiation dose. At 8.0 Gy radiation, dense p53 accumulation was observed in almost all of the basal cells at 4 h post-irradiation, and they were still detected in the basal cell layer at 24 h post-irradiation.

To determine whether or not radiation affects the biosynthetic rate of the p53 protein, cells were labeled with [^{35}S]-methionine in the methionine free media for 1 h or 4 h and immunoprecipitated with the p53 monoclonal antibody (Fig. 3). The newly synthesized p53 protein was weakly detected at 2.0 Gy radiation for 1 h post-irradiation. At 4 h post-irradiation, the newly synthesized p53 at 2.0 Gy radiation was approximately twice that of non-irradiated. However, the possibility that this increase in [^{35}S]-methionine labeled p53 may also result from its increase in stability during the 4 h post-irradiation time was not excluded in this study.

c-fos and cyclin D1 were transiently induced within 1 h post-irradiation and maximal c-fos induction was observed at 0.1 Gy radiation Since it is highly suggested that γ-radiation stimulates proto-oncogene expression and cell cycle regulatory protein, we examined whether or not cyclin D1, c-fos, pRb, and cdc-2 were induced or phosphorylated by γ-ray radiation (Fig. 4). The detergent extract of OM cells, which were labeled with [^{35}S] methionine for 1 h after radiation, was prepared and subsequently immunoprecipitated with specific antibodies to cyclin D1 and c-fos proteins (Fig. 4A). Cyclin D1 migrating at 36 kDa by SDS-PAGE was detected with a polyclonal antibody to human cyclin D1, whose biosynthesis was stimulated within 1 h after radiation at all radiation doses tested. Approximately a 5 fold induction was observed at 0.1-2.0 Gy radiation doses, but less induction (3.8 fold induction) was observed at 8.0 Gy radiation.

The c-fos protein, migrating at 67 kDa based on SDS-PAGE, was detected with a monoclonal antibody to human c-fos. Within 1 h post-irradiation, the strongest induction of c-fos was observed at a 0.1 Gy radiation dose, which corresponds to the 7.3 fold induction of the control. At the 0.5 Gy radiation dose, c-fos induction was approximately 2.8 fold more than the control. At 2.0 Gy and 8.0 Gy radiation, which are higher than LD_{50} (1.83 Gy), c-fos inductions were minimal. Cyclin D1 and c-fos induction turned out to be only transient since their induction went back to the basal level at 4 h post-irradiation.

To explore whether or not the phosphorylation pattern of cdc-2 and pRb were affected by radiation, the cdc-2 and pRb phosphorylation were also measured by Western analysis with antibodies to specific phospho-forms of cdc-2 and pRb proteins (Fig. 4B). Their phosphorylations were unaffected by radiation at all of the doses tested. Therefore, c-fos may be

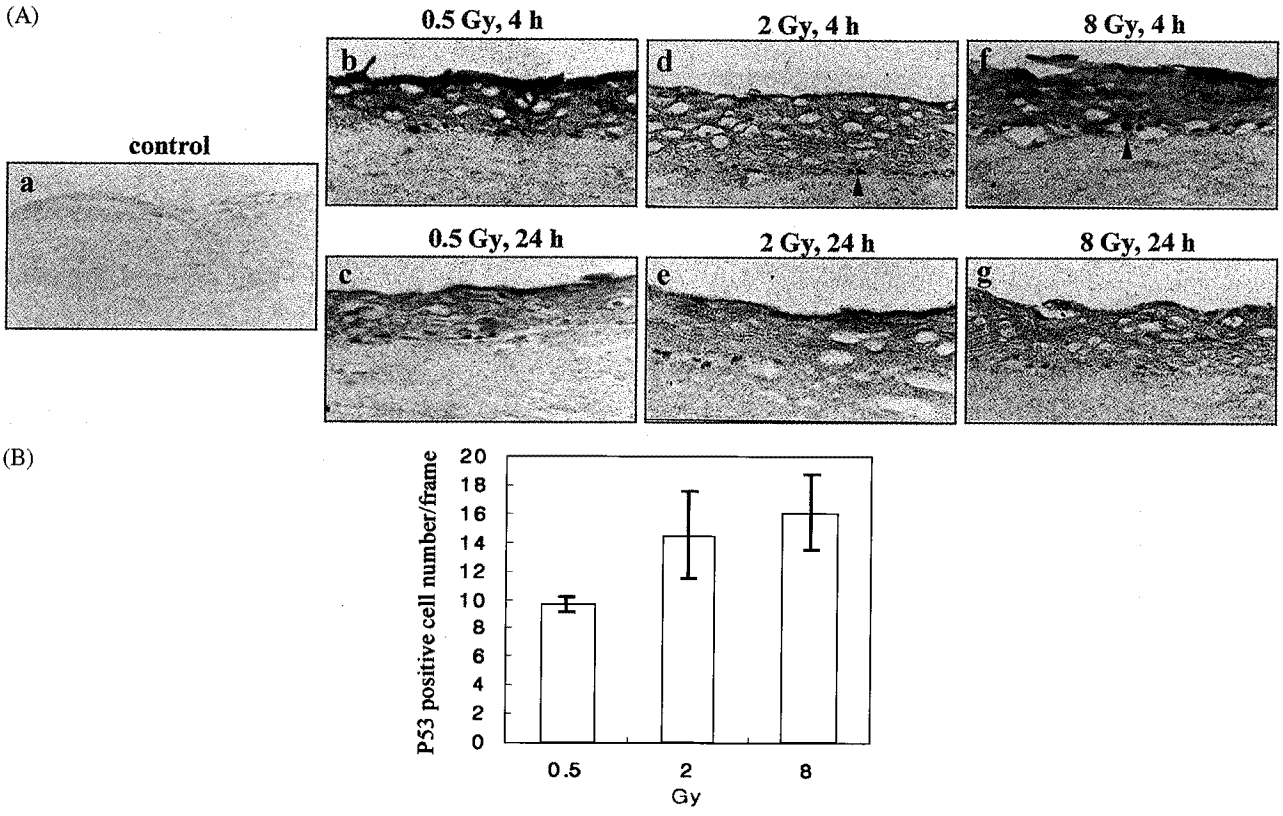

Fig. 2. Immunohistochemical staining of p53 accumulation within the architecture of the OM cells raft culture after irradiation. The γ-ray was irradiated on the OM cells raft culture at doses of 0 Gy (a), 0.5 Gy (b, c), 2.0 Gy (d, e), 8.0 Gy (f, g) and the OM cells raft culture were fixed in Carnoy's solution at 4 h (a, b, d, f) and at 24 h post-irradiation (c, e, g). Immnuohistochemical staining was carried out as described in Materials and Methods (▲ denote p53 nuclear accumulation) (A). Mean value of p53 positive cells counted from three different sections of the OM raft culture at 4 h post-irradiation time (B).

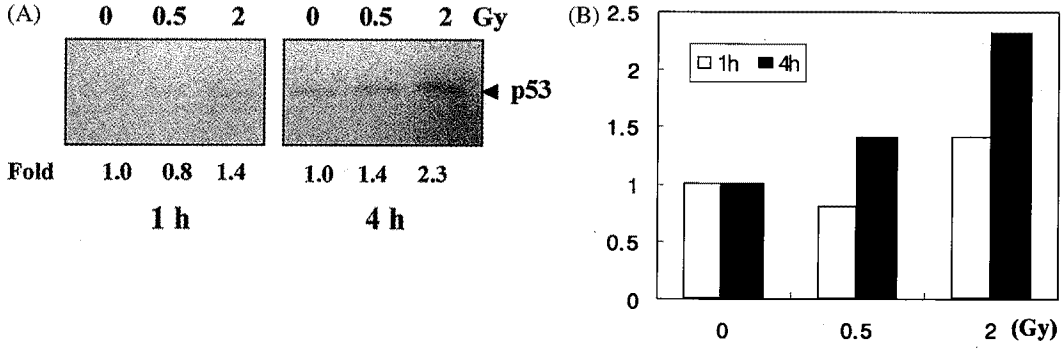

Fig. 3. Immunoprecipitation of newly synthesized p53 after irradiation in the OM cells. To detect the change of synthesized p53 for 1 h post-irradiation and 4 h post-irradiation, OM cells were continuously labeled with [^{35}S]-methionine in a methionin free media for 1 h or 4 h after irradiation. The p53 was immunoprecipitated (I.P) with specific antibody and detected with autoradiography (A). The p53 band intensity from the densitometer reading were represented as relative based on control intensity (B).

considered as one of the immediate early signaling molecules for the low dose radiation specific cellular response.

Radiation-induced reduction in EGFR tyrosine phosphorylation Since ionizing radiation, at clinically relevant doses, activates the EGF receptor in the A431 cell line, we investigated whether or not radiation stimulates phosphorylation of the EGF receptor in normal OM epithelial cells. Cells were extracted within 10 min after radiation, immunoprecipitated with the EGF receptor antibody, and subsequently immunoblotted with a phospho-tyrosine antibody (Fig. 5A). The basal level of the EGF receptor phosphorylation was clearly shown, which suggests that autocrine activation of the EGF receptor is present in OM cells. At a 0.1 Gy radiation, the EGF receptor phosphorylation was only slightly increased, but its phosphorylation was

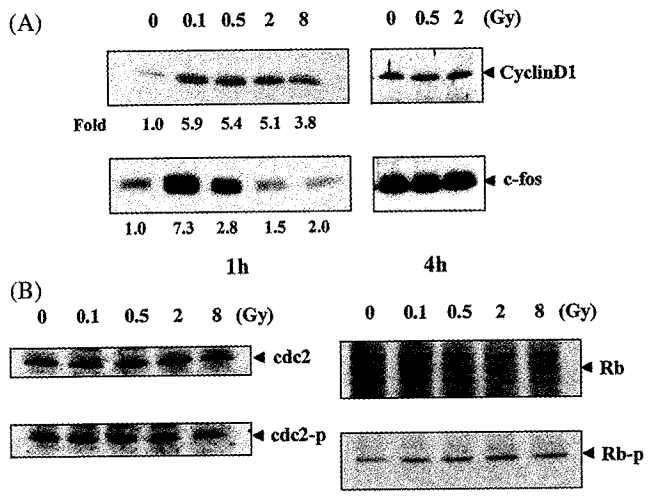

Fig. 4. Effect of γ-irradiation on expression of cyclin D1 and c-fos and phosphorylation pattern of cdc-2 and pRb. To detect the change of synthesized cyclin D1 and c-fos for 1 h post-irradiation and 4 h post-irradiation, OM cells were continuously labeled with [^{35}S]-methionine in a methionin free media for 1 h or 4 h after irradiation. Cyclin D1 and c-fos were immunoprecipitated with specific antibody and detected with autoradiography (A). Upon irradiation, the cell was immediately lysed and the same total protein (50 μg) were resolved by SDS-PAGE and immunoblotted with specific antibody of cdc2, Rb, phospho-cdc2 and phospho-Rb (B).

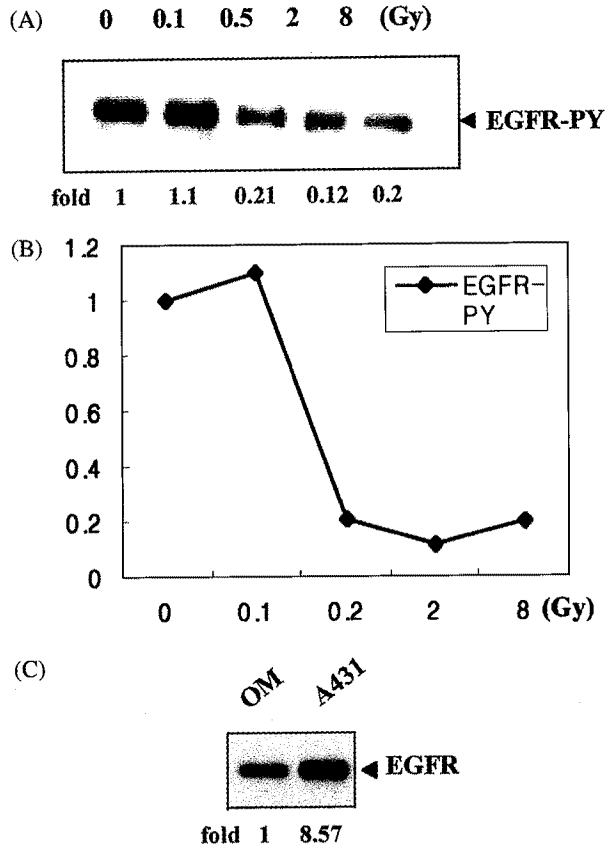

Fig. 5. Effect of γ-irradiation on EGFR phosphorylation. OM cells were irradiated at 0, 0.1, 0.5, 2.0 and 8.0 Gy and then harvested within 5 min. Cell lysates were immunopreciptated with a monoclonal antibody to human EGFR and immunoblotted with a polyclonal antibody to human phosphotyrosin (A). Values of EGFR phosphorylation from a densitometer reading were represented as relative values based on control intensity (B). In the A431 and OM cells, expression levels of EGFR were compared (C).

strongly reduced below the basal level at 0.5 Gy-8.0 Gy radiation doses. This result is in contrast to that of A431, which overexpresses the EGF receptor approximately 8.5 fold higher than normal oral mucosal cells (Fig. 5C). The discrepancy between A431 cells and OM cells may be due to the level of the EGF receptor at the cell surface. Therefore, the radiation-induced EGF receptor phosphorylation, or its dimerization observed in A431 by another group, may not be generalized to the radiation-induced cellular response of normal cells.

To examine whether or not Erk activation or deactivation was accompanied in the radiation-induced cellular response, we carried out a Western analysis of the same extracts applied for the EGF receptor phosphorylation assay (Fig. 6). Erk levels, or Erk phosphorylation, was unaffected by radiation within the 10 min post-irradiation time.

Discussion

In this study, we examined radiation-induced cellular responses from the viewpoint of radiation doses and post-irradiation times to evaluate radiation dose-response relationships in normal epithelial cells. Based on the cell viability evaluation, radiation-induced apoptosis continued up to 96 h post-irradiation at 2.0 Gy, or at a higher dose because the number of the viable cells continuously decreased up to 96 h post-irradiation. However, at lower radiation doses, the 48 h post-irradiation time may be adequate for estimation of the radiation sensitivity, since the radiation dose-response relationship was observed.

Nuclear accumulation of p53 was prominent at the basal cell layer of the OM raft culture, which corresponds to the proliferating cell layer. The p53 accumulation was apparent from 0.5 Gy radiation at 4 h post-irradiation and sustained up to 24 h post-irradiation. The number of p53 positive cells and their disappearance seem to be dependent on radiation doses. Therefore, the p53 positive cells that are probably represented as apoptotic cells (Schwartz *et al.*, 1997), or cells under DNA damage repair, may be considered as radiation-affected cells (Dulic *et al.*, 1994; Mallya and Sikpi, 1998). In this regard, the number of p53 positive cells at the given post-irradiation time may be applied for biological radio-dosimetry parameter. The radiation sensitivity data suggests that the p53 nuclear accumulation as a radio-dosimetry parameter may still be true up to a 96 h post-irradiation, since apoptotic cell death seems

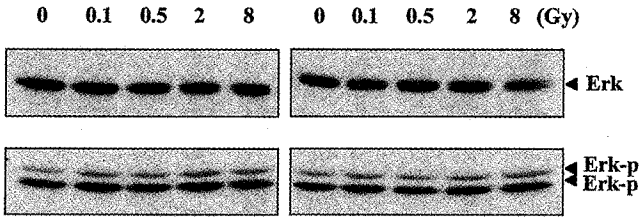

Fig. 6. Expression of Erk and phospho-Erk in the irradiated OM cells. Upon irradiation, the cell was immediately lysed, resolved by SDS-PAGE and detected by immunoblotting. Erk and phospho-Erk, using the same total protein (50 μg), were detected.

to be continued up to this time point (Komarova *et al.*, 1997). However, future study with more samples and at longer post-irradiation time points will determine whether or not p53 positivity shows a linear dose-response relationship or a stair-type relationship.

The radiation stimulated p53 biosynthesis at 4 h post-irradiation was in a dose dependent manner. Since p53 stability increases when it binds to DNA breakage (Beham *et al.*, 1997; Gottlieb *et al.*, 1997), the increase in [^{35}S]-methionine labeled p53, as shown in Fig. 3, may result from its increase in stability due to radiation-induced DNA damage (Zhan *et al.*, 1994). However, our study was unsuitable for distinguishing the two possibilities. This may be resolved by a nuclear run on assay or Northern blot analysis.

C-fos proto oncogene is specifically induced at a low dose radiation, as low as 0.1 Gy. The degree of c-fos induction was reversely correlated with radiation doses in the ranges of 0.1 to 8.0 Gy. This pattern of reverse dose-response relationship was also observed in EGF receptor deactivation. Since these two parameters were involved in the cellular signaling leading to cell proliferation (Hazzalin *et al.*, 1997; Reardon *et al.*, 1999), c-fos induction and lack of EGF receptor deactivation at 0.1 Gy may suggest that this dose may stimulate cell proliferation. This is often referred to as post-irradiation cell proliferation (Schmidt-Ullrich *et al.*, 1996), or low dose adaptive response (Shimizu *et al.*, 1999). Both the cyclin D1 and c-fos induction were prominent but transient, which suggests that these parameters may be valuable for the study of the radiation-induced cellular response rather than the radio-dosimetric parameter.

EGF receptor phosphorylation is one of the well-known phenomena observed in irradiated A431 (Goldkorn *et al.*, 1997; Carter *et al.*, 1998). However, OM cells, which are normal epithelial cells and express a much lower EGF receptor on the cell surface, markedly reduced the receptor phosphorylation upon γ-radiation. Approximately a 5-fold reduction in the EGF receptor phosphorylation by γ-radiation suggests that the EGF receptor activation is not involved in the radiation-induced cellular response in normal OM cells. Even though we were unable to provide direct evidence for this discrepancy, we speculated that the level of the EGF receptor on the surface may be one of the factors, since the EGF receptor level in A431 was 10 fold higher than that in normal OM cells. An unusually high EGF receptor on the surface may be susceptible for the radiation-induced receptor cross-link with neighboring receptors, which subsequently autophosphorylate cross-linked receptors to each other (Dent *et al.*, 1999). Considering that normal epithelial cells, which are present in a different cellular status than cancer cells, may respond to the ionizing radiation differently than the cancer cell line. Our results, which were primarily observed with cultured OM cells in the raft culture, may represent the response in normal epithelial tissue.

In spite of the EGF dephosphorylation, Erk deactivation was not accompanied. Sustained Erk activation may also be maintained by the activation of a variety of other signaling pathways, which might be turned on by radiation (Wilhelm *et al.*, 1997).

In conclusion, radiation-induced cellular responses are highly dependent on radiation doses and post-irradiation time as well as the cellular status. Among radiation specifically induced biomolecules, the p53 nuclear accumulation may be considered for the development of a useful marker for biological radio-dosimetry in normal epithelial tissue since its accumulation was sustained for a longer period and showed a dose response relationship. Specific c-fos induction at a low dose may also be an important finding in this study. It needs to be studied further for the elucidation of its connection with the low dose radio-adaptive response.

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