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Ionizing Radiation Induces a Stress Response in Primary Cultures of Rainbow Trout Skin

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Fish skin is very vulnerable to damage from physical and chemical pollutants because it is in direct contact with the aquatic environment. In this study, the effect of γ radiation on primary cultures of rainbow trout skin was investigated. Primary cultures containing two cell types, epidermal cells and goblet mucous cells, were exposed to doses ranging from 0.5–15 Gy ^{60}Co γ radiation. Expression of PCNA, c-myc and BCL2 was investigated as well as growth and levels of apoptosis and necrosis. Morphological and functional changes were also studied. The irradiated cultures showed evidence of a dose-dependent increase in necrosis and enhanced proliferation as well as morphological damage. In addition, mucous cell area was found to decrease significantly after irradiation. The study shows the value of these primary cultures as *in vitro* models for studying radiation effects. They provide an effective alternative to whole-animal exposures for radiation risk assessment. © 2004 by Radiation Research Society

INTRODUCTION

Fish skin is continuously exposed to and in intimate contact with the aquatic environment. The epidermis, which is covered with a layer of mucus that forms an additional barrier to potentially harmful substances, protects the animal against external effects (1). Unlike the gill epithelium, the impact of environmental pollutants on this tissue has received little attention, although it is metabolically very active and responds rapidly to a wide variety of stressors (2–9). It is an important route of entry for waterborne radioisotopes in the environment and is likely to show effects of direct contact with contaminated water containing radio-

active substances due to releases from nuclear power stations.

There are few reports in the literature relating to the effects of low levels of ionizing radiation on fish. Much of the work details the effects of very high doses of radiation, which are not relevant for environmental protection applications.

While there is some literature on fish radiobiology, much of the data are for medaka, *Oryzias latipes*, which has been used as a model system for the study of germ cell mutagenesis after exposure to ionizing radiation (10). In addition to work done on medaka, Knowles (11) reported an immune response in rainbow trout exposed to chronic γ radiation for 246 days after fertilization. The total accumulated dose over the whole period was 5–25 Gy. Goncharova *et al.* (12) described genetic effects of low-dose chronic irradiation (0.4–5.5 μGy per day) in carp from ponds radiocontaminated by the Chernobyl accident. There are some studies on the effects of UV radiation on fish. Effects of UV radiation on the eye lens of rainbow trout have been reported (13, 14). O'Reilly and Mothersill (15) documented that a fish cell line, EPC, was more sensitive to UVA and UVB radiation than were the human cell lines HPV-G and HaCaT. Cellular changes characteristic of sunburn damage have been reported in sole, *Solea solea*, exposed to UVB radiation, whereas turbot, *Scophthalmus maximus*, with its heavily pigmented skin, was found to be unaffected by similar doses (16). UVB-radiation-mediated increases in oxidative stress in muscle and skin of zebrafish, *Brachydanio rerio*, have also been documented (17).

Tissue and cell culture systems are extremely useful for studying the behavior of cells independent of systemic control (18), and fish cells have been used *in vitro* for the assessment of the toxicity of environmental pollutants to aquatic biota (19). This laboratory developed a primary culture model system of rainbow trout skin (20) and has studied the effects of various environmental agents on this *in vitro* model. Lyons-Alcantara *et al.* (3, 4) investigated the effects of sublethal doses of cadmium and reported growth reduction, increased apoptosis, increased glycogen deposits and melanization, and increased heat-shock protein expres-

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sion. Reduction in growth, decreases in mucous cell number, and necrosis were reported after exposure to nonoxynol, copper and prochloraz (6, 7). Kilemade and Mothersill (8, 9) reported inhibited epidermal cell proliferation, necrotic epidermal cell death, and increased heat-shock protein expression after exposure to 2,4-dichloroaniline.

Apart from their critical importance in aquatic food webs, fish, as lower vertebrates, are an important species for mechanistic studies of radiation biology, because an understanding of the evolution of protective mechanisms may be gained. The aim of the present study is to investigate the effect of γ radiation on primary cultures of rainbow trout skin. In an effort to understand mechanisms of damage response in fish, end points relating to apoptosis, necrosis and proliferation were examined in relation to dose. Morphological and functional changes were also studied.

MATERIALS AND METHODS

Primary Culture of Rainbow Trout Skin

Healthy rainbow trout (*Oncorhynchus mykiss*), 35–50 g, were obtained from a commercial fish farm in Co. Tipperary, Ireland. Rainbow trout parr were used in these experiments. The animals were killed humanely by a qualified veterinary practitioner (ML), and the procedure was approved by the Dublin Institute of Technology ethics committee. Epithelial cell growths were initiated from the skin explants using a technique described elsewhere (3, 20). Briefly, fish were aseptically dissected immediately after death. Care was taken to avoid contamination or damage to the outermost epidermal cells. Skin tissue was cleaned of muscle and fibrous tissue to avoid fibroblastic growth and cut into 2-mm² pieces while immersed in sterile PBS or growth medium. It was not necessary to digest the tissue with enzymes. Single pieces of tissue were plated in the center of 25-cm² Falcon flasks containing 2 ml RPMI 1640 growth medium containing 13% fetal calf serum (Gibco Biocult, Scotland), 7% horse serum (Gibco Biocult), 100 mIU insulin (Novo Nordisk, Copenhagen), 1 μ g/ml hydrocortisone (Glaxo), 50 IU/ml penicillin, 50 μ g/ml streptomycin solution (Gibco Biocult) and 1 μ g/ml fungizone/amphotericin B (Gibco). Cultures were incubated in a refrigerated incubator (Leec, Nottingham, UK) at 19°C in an atmosphere containing 95% air/5% CO₂.

Irradiation

Explant cultures were irradiated *in situ* on the culture flasks 5 days after explantation. The dose was delivered at room temperature using a ⁶⁰Co teletherapy unit at a flask-to-source distance of 80 cm. Under these conditions, the dose rate was approximately 1.9 Gy/min during the experiments. The control cultures were removed from the incubator and brought to the ⁶⁰Co teletherapy unit with the irradiated cultures but were not irradiated. After irradiation, the cultures were replaced in the refrigerated incubator and maintained there until they were processed 48 h later. The doses used for these experiments were 0.5, 5, 10 and 15 Gy.

Growth Measurement

Cultures derived from the explants were fixed in 10% formalin 2 days after irradiation and were stained with Harris hematoxylin. Epithelial cell outgrowths were recorded using a transparent sheet of 1-mm² grid paper. The number of 1-mm² grids covered by the stained explant was counted.

Immunocytochemistry

An avidin-biotin immunoperoxidase method was used to visualize BCL2, c-myc and PCNA antibodies. Fish skin primary cultures, fixed 2

days after irradiation in 10% formalin for 5 min, were transferred to PBS and assayed immediately. The explant outgrowth was processed *in situ* on the flask bottom to enable spatial distribution to be related to the type of cell and degree of differentiation. The expression system used was a Vectastain ABC kit (Vectastain Corporation Burlingame, CA), which follows an indirect immunoperoxidase technique. Endogenous peroxidase enzyme activity was quenched using 3% hydrogen peroxide. Cultures were then incubated with normal blocking serum. The outgrowths were exposed to monoclonal anti-mouse PCNA (clone PC-10), BCL2 (Dako Ltd, Uden, The Netherlands), and c-myc (clone 9E10, a gift from G. Evan, then at ICRF, London) antibodies. Cultures were incubated with the Vectastain ABC reagent (avidin-biotin peroxidase solution). Diaminobenzidine tetrachloride (DAB) was used to visualize the reaction, and cultures were lightly counterstained with Harris hematoxylin. A positive reaction gives a brown product. Positive controls were from a positive human tissue block, obtained from the Cell Pathology Laboratory, Dublin Institute of Technology. Negative controls were cultures to which all reagents apart from the primary antibody were added. Positive and negative controls were run with every immunocytochemistry run-to-run to correct for run variability. Due to the small amounts of material (approximately 0.4 mg total cells) and the desirability of using *in situ* approaches, it was not possible to check expression levels using Western blot analysis.

Quantification of Protein Expression, Apoptosis, Necrosis, Mucous Cell Number and Mucous Cell Area

Numbers of cells positive for PCNA, BCL2 and c-myc and numbers of apoptotic and necrotic cells and mucous cells were scored along transects drawn at right angles to each other through the middle of the outgrowth so that both the edge and the middle areas of the outgrowth were included in the count. Over 1000 cells were scored in each culture. Cells were scored as necrotic if they displayed swelling of the cytoplasm, organelle dissolution, plasma membrane rupture, or vacuolization. Cells were defined as apoptotic if they displayed evidence of two or more of the following: cell volume shrinkage and pyknotic nucleus (chromatin condensation), blebbing of the cytoplasm, nuclear fragmentation and formation of apoptotic bodies (21). TEM was used to confirm the presence of apoptotic cells. Mucous cells were recognized by their basophilic cytoplasm, basally located nuclei, and the accumulation of mucus secretory granules that filled and distended their apex to give the characteristic goblet shape (22). Mucous cell areas were measured using a Leica Q500MC image analyzer (Leica Microsystems, Germany). At least 300 mucous cells measured along transects drawn at right angles to each other were scored per dose.

Transmission Electron Microscopy

After the cultures were washed in buffer, they were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 1 h, post-fixed in 1% OsO₄ in 0.1 M phosphate buffer for a further hour, dehydrated in ascending grades of ethanol, and subsequently embedded in epoxy resin. Thick sections (2 μ m) were cut *en face* with a glass knife, stained with 0.1% toluidine blue, and examined under the light microscope. Thin sections (80 nm) were then cut from the same block face with a diamond knife, mounted on 100 mesh unsupported copper grids, stained with uranyl acetate and lead citrate, and examined using a JEOL 2000 transmission electron microscope. Three areas of the cell outgrowth from three replicate cultures were examined at the ultrastructural level.

Statistical Analysis

All experiments were repeated three times, and within each experiment cultures were set up in triplicate. Results are expressed as the means \pm standard errors. Significance was determined using the *t* test.

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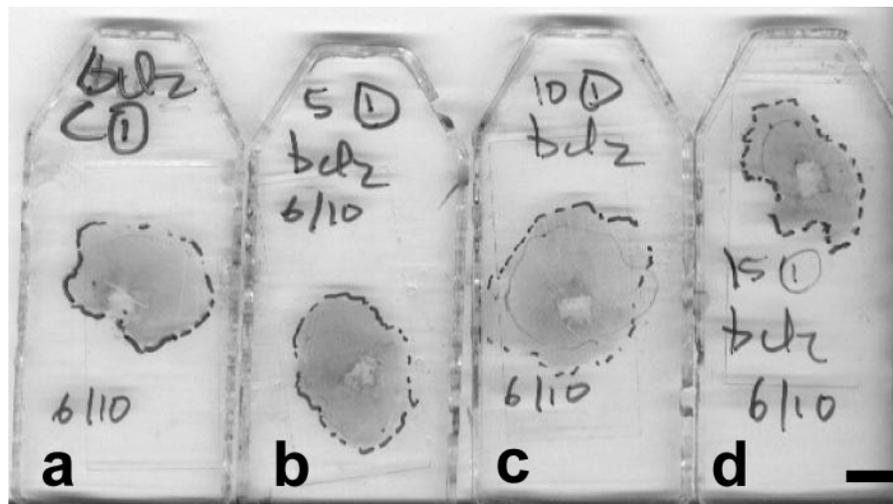


FIG. 1. Outgrowth areas from primary cultures of rainbow trout skin exposed to (a) 0 Gy (b) 5 Gy, (c) 10 Gy and (d) 15 Gy γ radiation. Bar = 1 cm.

RESULTS

Effect of Radiation on Growth

Figure 1 and Table 1 show the outgrowth area of control and irradiated rainbow trout skin primary cultures. No significant difference was observed between control cultures and those exposed to radiation.

Ultrastructural Morphology

The epidermis of fish skin is a stratified squamous epithelium. Mucus-secreting cells usually originate in the middle layers of the epidermis and increase in size as they approach the surface. Skin from rainbow trout showed polygonal epithelial cells with closely opposed plasma membranes and centrally placed nuclei and occasional oval-shaped mucous cells with basally located nuclei (Fig. 2a). Numerous desmosome junctions and intermediate filaments were present, and free ribosomes were abundant in the cytosol (Fig. 2b).

Primary cultures of rainbow trout skin showed polygonal epithelial cells with centrally placed nuclei. Oval-shaped

mucous cells were also observed (Fig. 2c). Desmosome junctions and intermediate filaments were present, confirming the epithelial nature of the cells (Fig. 2d, e).

Primary cultures of rainbow trout skin exposed to 0.5 and 5 Gy γ radiation showed normal polygonal epithelial cells with occasional mucous cells. No changes in morphology were seen at these doses.

After the higher doses of 10 and 15 Gy, cells with irregular elongated nuclei and increased vacuolization were observed. Desmosome junctions were intact and intermediate filaments were present. Apoptotic cells showing condensed chromatin and a shrunken morphology were also seen (Fig. 2f). These were quantified using light microscopy (Table 1).

Apoptosis

Figure 3a shows the percentage of cells with morphological changes indicative of apoptosis in control and irradiated primary skin cultures. The results show that apoptosis is not an important response mechanism in this system.

Necrosis

Figure 3a shows the percentage of cells with morphological evidence of necrosis in control and irradiated primary skin cultures. There was a significant increase in the numbers of necrotic cells after irradiation.

Mucous Cells

Mucous cells were present in the controls and were distributed all over the outgrowths (Fig. 2c). Table 1 shows the effect of radiation on mucous cell numbers in outgrowths exposed to radiation. Radiation exposure did not significantly affect the percentage of mucous cells. Figure 3b shows the effect of radiation on mucous cell area. A

TABLE 1
Effect of Ionizing Radiation on Growth, Number of Mucous Cells, and Expression of PCNA in Primary Cultures of Rainbow Trout Skin

| Dose | Outgrowth area (mm ²) | Percentage mucous cells | Percentage PCNA-positive cells | |
|-------|-----------------------------------|-------------------------|--------------------------------|----------------|
| | | | Very dark staining | Weak staining |
| 0 Gy | 210 \pm 55 | 2.4 \pm 0.5 | 13.0 \pm 1.0 | 13.0 \pm 3.1 |
| 5 Gy | 193 \pm 75 | 2.3 \pm 0.7 | 11.5 \pm 0.5 | 14.0 \pm 6.0 |
| 10 Gy | 235 \pm 65 | 2.2 \pm 0.8 | 17.3 \pm 5.2 | 11.7 \pm 1.8 |
| 15 Gy | 213 \pm 35 | 1.9 \pm 0.4 | 22.0 \pm 7.0 ^a | 15.0 \pm 1.0 |

^a $P < 0.05$.

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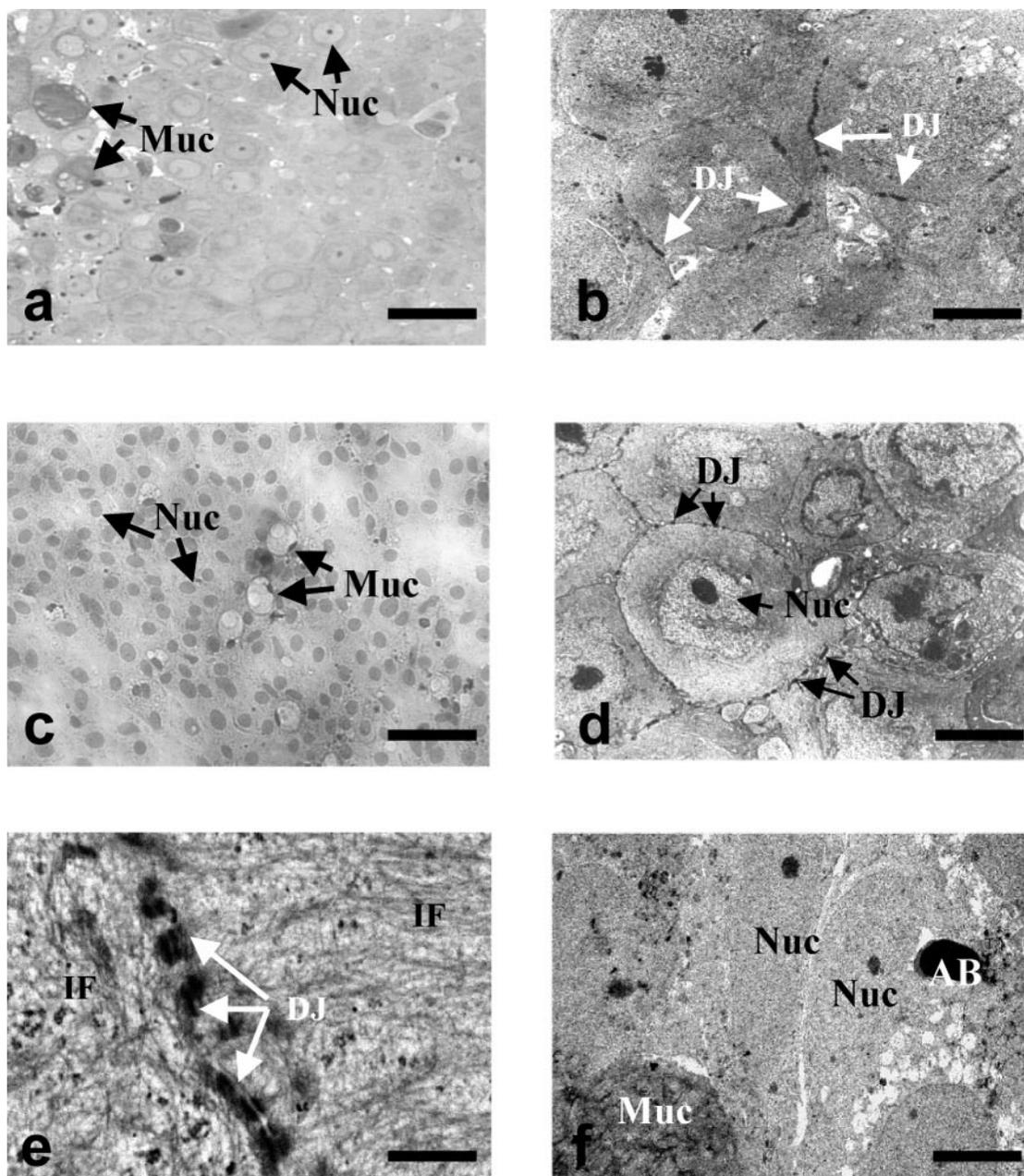


FIG. 2. Panel a: Skin from control rainbow trout showing polygonal epithelial cells with closely opposed plasma membranes and centrally placed nuclei (Nuc). Oval-shaped mucous cells (Muc) were observed occasionally. Bar = 20 μm . Panel b: Skin from control rainbow trout showing desmosome junctions (DJ). Free ribosomes are abundant in the cytosol. Bar = 5 μm . Panel c: Primary culture from rainbow trout skin showing polygonal epithelial cells with centrally placed nuclei (Nuc). Oval-shaped mucous cells (Muc) were also observed. Bar = 20 μm . Panel d: Primary culture from rainbow trout skin showing a regular nucleus (Nuc) and desmosome junctions (DJ). Bar = 5 μm . Panel e: Primary culture from rainbow trout skin showing intermediate filaments (IF) and desmosome junctions (DJ). Bar = 1 μm . Panel f: Primary culture from rainbow trout skin exposed to 10 Gy γ radiation showing elongated nuclei (Nuc), an apoptotic body (AB), and a mucous cell (Muc). Bar = 5 μm .

significant dose-dependent reduction in mucous cell area was observed after irradiation.

Expression of PCNA, c-myc and BCL2

Table 1 shows the percentage of PCNA-positive cells in control and irradiated skin primary cultures. Both very darkly stained (equal to or more intense than the positive

control) and weakly stained (less intense than the positive control) PCNA-positive cells were scored. The percentage of weakly stained cells did not change significantly with increasing radiation dose, but the percentage of darkly stained cells increased significantly at 10 and 15 Gy.

Figure 3c shows the percentage of c-myc-positive cells in control and irradiated cultures. The percentage of c-myc-

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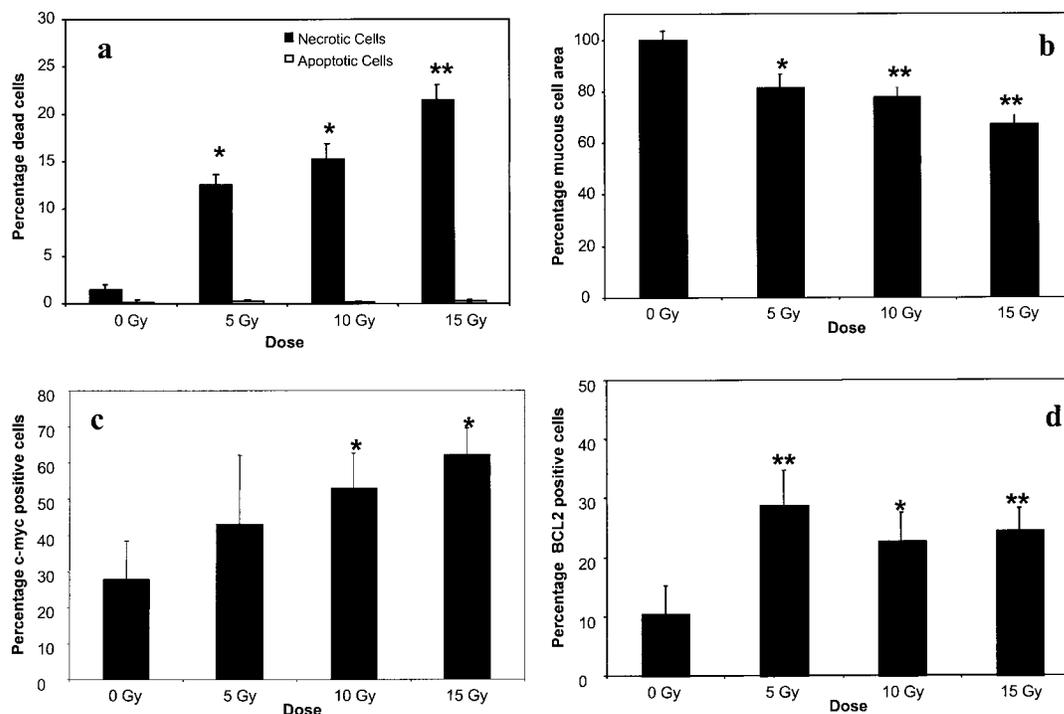


FIG. 3. Panel a: Percentages of necrotic and apoptotic cells in rainbow trout skin primary cultures exposed to radiation. Data are presented as means \pm standard deviations. * $P < 0.01$, ** $P < 0.001$. Panel b: Percentage reduction in mucous cell areas compared to controls in rainbow trout skin primary cultures exposed to radiation. Data are presented as means \pm standard deviations. * $P < 0.05$, ** $P < 0.005$. Panel c: Percentage c-myc-positive cells in rainbow trout skin primary cultures exposed to radiation. Data are presented as means \pm standard deviations. * $P < 0.01$. Panel d: Percentage BCL2-positive cells in rainbow trout skin primary cultures exposed to radiation. Data are presented as the means \pm standard deviations. * $P < 0.05$, ** $P < 0.01$.

positive cells was found to increase with increasing radiation dose.

Figure 3d shows the percentage of BCL2-positive cells in control and irradiated cultures. An increase in the percentage of BCL2-positive cells was observed after radiation exposure.

DISCUSSION

This study showed increased necrosis and enhanced expression of PCNA, c-myc and BCL2 in primary cultures of rainbow trout skin exposed to γ radiation. Morphological changes and functional changes involving mucous cells were also observed. No change in outgrowth area or in the number of apoptotic cells was found after irradiation.

Cell proliferation was assessed using immunocytochemical staining of PCNA. Ortega *et al.* (23) found that PC-10, the clone used in the present study, gave the best results out of three PCNA antibodies in three fish species. In the present study, cells showed both weak and intense nuclear staining of PCNA. The intense staining observed was indicative of cells in the S phase of the cell cycle, whereas the weakly stained fraction was indicative of noncycling cells (24, 25). Only cells with intensely positive nuclei, reflecting newly synthesized PCNA from actively cycling cells, showed a dose-dependent response to radiation. The

increased expression of PCNA with increasing dose indicates enhanced proliferation after irradiation. The concomitant increase in the number of necrotic cells could explain the similar outgrowth areas observed in the control and irradiated cultures.

An increase in the expression of both c-myc and BCL2 was observed, with no change in the number of apoptotic cells. Aberrant expression of c-myc is known to cause apoptosis, but c-myc is also essential for cell proliferation (26, 27). It is thought that every cell that enters the cell cycle invokes a suicide pathway that must be continuously suppressed by signals from the immediate cellular environment for the proliferating cell to survive (27). In this case, it appears that increased expression of BCL2 inhibits the removal of damaged cells by apoptosis. C-myc and BCL2 family proteins have been identified previously in rainbow trout and other fish (28–31).

The pattern of expression of apoptosis and necrosis found in this study is similar to that seen by our group for both human uroepithelium and prawn hematopoietic tissue after exposure to ionizing radiation (32, 33). BCL2 and c-myc are both expressed, and necrosis appears to be the predominant mechanism of cell death. Primary cultures from fish skin and human uroepithelium appear to have similar sensitivities to radiation, whereas cultures from the prawn hematopoietic tissue appear to be considerably more

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sensitive. Current investigations involving primary cultures of rainbow trout pronephros (the main hematopoietic tissue) suggest that when like tissues are compared, the trout is more radioresistant than the prawn (34).

Morphological changes were also observed after irradiation. Changes in nuclear shape were observed as well as increased vacuolization. Nuclear irregularities may be a consequence of an increase in metabolic activity or a disturbance of mitosis. Rupture of cellular organelles leading to vacuolization may be due to increased water inflow caused by a disturbance of ionic concentration (35).

A functional change involving the mucous cells was also observed. Although no change in the numbers of mucous cells was seen, the mucous cell area was found to decrease significantly after irradiation. This may have been due to the mucous cells releasing their contents onto the cell culture surface in response to the radiation-induced stress. An increase in mucus production is a well known response to stress. Dowling and Mothersill (7) reported a decrease in the number of mucous cells in primary rainbow trout cultures after exposure to copper and prochloraz and also showed increased PAS staining for mucus in the exposed cultures. This stimulation of mucous secretion in rainbow trout skin was similar to that reported previously for fish exposed to ecological stressors (36).

This study has shown effects of γ radiation on primary cultures of rainbow trout skin and shows the value of primary cultures as an *in vitro* model for radiation biology studies of complex organisms in their natural environment. *In vitro* studies can be used as a very effective alternative to whole animal exposures. Kilemade and Mothersill (37) reported good correspondence between *in vivo* and *in vitro* results for identifying toxic effects in fish skin. *In vitro* studies enable the response to a single agent to be examined in a single tissue, allowing mechanistic studies and dose-response studies to be performed in a controlled way. A key point is to determine whether function of the tissue in the fish is impaired by radiation exposure. While death of the cells is a key determinant of dose effect in human radiobiology, fish or other species could die as a result of impaired function of defense systems, and that "indirect" effect could have consequences that are not readily detected using conventional assays for radiation damage.

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