Oxidative stress in cells exposed to low levels of ionizing radiation

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Abstract

The ability of medium from γ-irradiated cells to induce early events in the apoptotic cascade, such as the mobilization of intracellular calcium, loss of mitochondrial membrane potential and increased levels of reactive oxygen species, in unirradiated cells was investigated. Medium from irradiated human keratinocytes was harvested and transferred to unirradiated keratinocytes. Intracellular calcium levels, mitochondrial membrane potential and the level of reactive oxygen species were all monitored for a period of 24 h following medium transfer. Rapid calcium fluxes (within 30 s), loss of mitochondrial membrane potential and increases in reactive oxygen species (from 6 h after medium transfer) were observed. There was no significant

Key words: apoptosis, intracellular calcium, irradiated cell conditioned medium, mitochondrial membrane potential, reactive oxygen species.

Abbreviations used: HPV, human papillomavirus; ICCM, irradiated cell conditioned medium; LET, linear energy transfer.

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difference between the effects of medium generated by cells irradiated at 0.5 Gy or 5 Gy. The data suggest that a signal that leads to apoptosis is released from cells undergoing radiation-induced oxidative stress.

Introduction
It has been suggested that a factor or signal is released by irradiated cells that can affect unexposed cells in the field following high linear energy transfer (LET) radiation [1–4]. Cells that are not even in the field can be affected by medium harvested from irradiated cells exposed to low doses of low LET radiation [5–8]. Our group has shown 'bystander' effects when the medium from epithelial cells irradiated with γ-rays is transferred to cultures that have not been irradiated [5,6]. This irradiated cell conditioned medium (ICCM) can reduce clonogenic survival and increase the incidence of apoptosis in cells that have never sustained any irradiation. The effect is dependent on the cell number present at the time of irradiation, strongly suggesting the production of a molecule by the irradiated cell. The factor is stable when frozen to -20 °C, but destroyed on heating to 70 °C. Holding the cells on ice during and after irradiation prevents production of the bystander effect in cells receiving the ICCM [6]. In addition, we have shown previously that treatment with the antioxidants L-lactate and L-deprenyl prevented the bystander effect in ICCM-exposed HPV-G cells [9]. Treatment with cyclosporin A, which inhibits the collapse of the mitochondrial membrane potential, or with an interleukin-1β-converting enzyme inhibitor (Ac-Tyr-Val-Ala-Asp-Cmk), which inhibits selected caspases involved in the apoptotic cascade, also reduced or prevented the bystander effect in these cells.

These data suggest that initiating events in the apoptotic cascade are induced in unirradiated cells by a signal produced by irradiated cells. To investigate this, it was decided to study the ability of ICCM to induce early events in the apoptotic cascade. ICCM generated from immortalized human keratinocytes was added to keratinocytes that had never been irradiated. The effect was monitored using mobilization of intracellular calcium, loss of mitochondrial membrane potential and increased levels of reactive oxygen species as markers of apoptosis over a 24 h period after exposure. All these events have been clearly linked with the induction of apoptosis [10–13].

Materials and methods
A human keratinocyte cell line supplied as a gift by J. Di Paolo (NIH, Bethesda, MD, U.S.A.) was used. This line was originally immortalized by transfection with the human papillomavirus (HPV) 16 [14]. It is p53-null due to expression of E6 protein by the virus, but grows in culture to form a characteristic monolayer of cobblestone-like keratinocytes. These display contact inhibition and gap junctional intercellular communication.

HPV keratinocytes were cultured in Dulbecco's modified Eagle's medium/F12 (1:1, v/v) containing 7% (v/v) foetal calf serum, 5 ml of penicillin/streptomycin solution, 25 mM Heps buffer and 1 μg/ml cortisol (all from Gibco Biocult Ltd, Irvine, Scotland, U.K.), and were maintained in an incubator at 37 °C in an atmosphere of 5% CO2 in air. Subculture was routinely performed using a 1:1 (v/v) solution of 0.25% trypsin and 1 mM EDTA in Earle’s balanced salt solution at 37 °C.

Cultures were sealed and irradiated at room temperature using a cobalt 60 teletherapy unit delivering approx. 2.0 Gy/min during the time period of these experiments. The source-to-flask distance was 80 cm and the field size was 30 cm x 30 cm. Flasks were returned to the incubator immediately after irradiation.

The technique used for medium transfer has been described in detail by Mothersill and Seymour [5]. Briefly, medium was poured off donor flasks 1 h after irradiation. The medium was filtered through a 0.22 μm filter to ensure that no cells were still present in the transferred medium. Culture medium was then removed from the flasks designated to receive irradiated medium, and the filtrate was added immediately to these recipient flasks. A medium change of unirradiated but similarly filtered medium from unirradiated donor flasks was given to controls at the same time. Standard plating efficiency controls were also set up. There was never a significant difference between these two controls. Standard clonogenic survival points following irradiation were also always included, with and without a medium change at the appropriate time. No effect of changing the medium was found. The donor medium generated as described here is referred to as ICCM.

Intracellular calcium levels were measured using two visible-wavelength calcium-sensitive dyes, Fluo 3 and Fura Red. Fluo 3 exhibits an
increase in green fluorescence upon binding to calcium, whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The Fluo 3/Fura Red ratio is a good indication of intracellular calcium levels [15]. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₃HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM Hepes (pH 7.4). Cells were loaded with the calcium-sensitive dyes by incubation with 3 μM Fluo 3 and 3 μM Fura Red acetoxymethyl esters for 1 h in the buffer at 37 °C. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm, and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Bio-Rad 1024 confocal microscope.

Mitochondrial membrane potential was measured using rhodamine 123, a green fluorescent dye that accumulates in active mitochondria with high membrane potentials [16]. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₃HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM Hepes (pH 7.4). Cells were loaded with 5 μM rhodamine 123 for 30 min in the buffer at 37 °C. Subsequently, the cultures were washed three times with buffer. Rhodamine 123 was excited at 488 nm, and fluorescence emission at 525 nm was recorded using a Bio-Rad 1024 confocal microscope.

Induction of reactive oxygen species was measured using 2,7-dichlorofluorescein diacetate, which emits green fluorescence when oxidized by reactive oxygen species [17]. Cultures were washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₃HPO₄, 1 mM CaCl₂, 1 mM MgCl₂ and 25 mM Hepes (pH 7.4). Cells were loaded with 5 μM 2,7-dichlorofluorescein diacetate for 30 min in the buffer at 37 °C. Subsequently, the cultures were washed three times with buffer. 2,7-Dichlorofluorescein diacetate was excited at 488 nm, and fluorescence emission at 525 nm was recorded using a Bio-Rad 1024 confocal microscope.

Measurements are presented as means ± S.E.M. of three independent experiments, each performed in triplicate. Significance of differences was determined by a Student's unpaired t-test, and the differences were considered significant at \( P < 0.05 \).

**Results and discussion**

Rapid calcium fluxes (within 30 s) were observed following addition of ICCM. Figure 1 shows a rapid and transient increase in calcium levels following addition of 0.5 Gy ICCM. There was no significant difference between the responses to 0.5 Gy ICCM and 5 Gy ICCM. There was no change in intracellular calcium levels following addition of medium from unirradiated cells (0 Gy ICCM) or of control medium.

Mitochondria with high membrane potentials were observed in control cells and in cells treated with 0 Gy ICCM. No change in mitochondrial membrane potential was observed at 30 s or 1 h following addition of medium from irradiated cells, but a decrease in fluorescence and more unspecific staining was observed at 6, 12 and 24 h after addition (Table 1), suggesting a decrease in mitochondrial membrane potential. There was no

**Figure 1**

Intracellular calcium levels in HPV-G cells after addition of medium from unirradiated cells (0 Gy ICCM) or from irradiated cells (0.5 Gy ICCM)

ICCM was added at the time indicated by the arrow. The ratio of fluorescence emissions from the calcium-sensitive dyes Fluo 3 and Fura Red provides an indication of intracellular calcium levels.

**Table 1**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0 Gy</th>
<th>0.5 Gy</th>
<th>5 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 s</td>
<td>62.56±3.58</td>
<td>60.44±4.32</td>
<td>64.11±4.88</td>
</tr>
<tr>
<td>1 h</td>
<td>61.23±3.76</td>
<td>66.9±2.85</td>
<td>63.29±3.55</td>
</tr>
<tr>
<td>6 h</td>
<td>60.33±2.99</td>
<td>20.65±0.89</td>
<td>20.78±0.96</td>
</tr>
<tr>
<td>12 h</td>
<td>62.96±3.80</td>
<td>22.77±1.44</td>
<td>21.86±1.39</td>
</tr>
<tr>
<td>24 h</td>
<td>60.45±2.55</td>
<td>20.79±1.22</td>
<td>20.93±0.90</td>
</tr>
</tbody>
</table>
significant difference between the responses to 0.5 Gy ICCM and 5 Gy ICCM.

When monitored for reactive oxygen species, very little fluorescence was observed in control cells or cells treated with medium from un-irradiated cells (0 Gy ICCM). Similarly, very little fluorescence was observed at 30 s following addition of medium from irradiated cells, but an increase in fluorescence was observed at 1, 6, 12 and 24 h after addition (Table 2), suggesting an increase in reactive oxygen species. There was no significant difference between the responses to 0.5 Gy ICCM and 5 Gy ICCM.

A decrease in clonogenic survival and an increase in apoptotic cells were also observed following addition of ICCM.

The mechanisms by which the bystander signal is produced and transduced are largely unknown. There is some evidence that apoptotic death is a prominent feature of cultures demonstrating bystander effects, and this is supported by the data presented in this paper. Kroemer et al. [11] showed that one of the first observable effects in the pathway leading to apoptosis is a change in the membrane permeability in the mitochondria, which resulted in the release of apoptosis-inducing factor from the mitochondria. Mitochondrial depolarization is thought to be associated with the early stage of apoptosis. Changes in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition transition pores that play a central role in apoptosis [12]. Intracellular calcium fluxes are also thought to be involved in the induction of apoptosis [10]. An increase in reactive oxygen species has also been linked to the initiation of the apoptotic cascade [13].

In conclusion, the present study suggests that a signal leading to apoptosis is released from cells undergoing oxidative stress induced by y-irradiation.

Table 2

<table>
<thead>
<tr>
<th>Time</th>
<th>Cells showing fluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Gy</td>
</tr>
<tr>
<td>30 s</td>
<td>7.32±0.34</td>
</tr>
<tr>
<td>1 h</td>
<td>6.99±0.45</td>
</tr>
<tr>
<td>6 h</td>
<td>6.59±0.36</td>
</tr>
<tr>
<td>12 h</td>
<td>7.08±0.41</td>
</tr>
<tr>
<td>24 h</td>
<td>6.95±0.25</td>
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References

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