Distinct Cellular Calcium Metabolism in Radiation-sensitive RKO Human Colorectal Cancer Cells

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INTRODUCTION

Radiation therapy for variety of human solid tumors utilizes mechanism of cell death after DNA damage caused by radiation. In response to DNA damage, cytochrome c was released from mitochondria by activation of pro-apoptotic Bcl-2 family proteins, and then elicits massive Ca²⁺ release from the ER that lead to cell death. It was also suggested that irradiation may cause the deregulation of Ca²⁺ homeostasis and trigger programmed cell death and regulate death specific enzymes. Thus, in this study, we investigated how cellular Ca²⁺ metabolism in RKO cells, in comparison to radiation-resistant A549 cells, was altered by gamma (γ)-irradiation. In irradiated RKO cells, Ca²⁺ influx via activation of NCX reverse mode was enhanced and a decline of [Ca²⁺]i via forward mode was accelerated. The amount of Ca²⁺ released from the ER in RKO cells by the activation of IP₃ receptor was also enhanced by irradiation. An increase in [Ca²⁺]i via SOCI was enhanced in irradiated RKO cells, while that in A549 cells was depressed. These results suggest that γ-irradiation elicits enhancement of cellular Ca²⁺ metabolism in radiation-sensitive RKO cells yielding programmed cell death.

Key Words: A549 cells, Inositol-1,4,5-triphosphate receptors, Na⁺-Ca²⁺ exchanger, RKO cells, Store-operated Ca²⁺ influx

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ABBREVIATIONS: RKO cells, RKO Human Colorectal Cancer Cells; [Ca²⁺], intracellular Ca²⁺ activity; NCX, Na⁺-Ca²⁺ exchanger; IP₃, Inositol-1,4,5-triphosphate; SOCI, Store-operated Ca²⁺ influx; PMCA, plasma membrane Ca²⁺-ATPase; SERCA, sarco/endo-plasmic Ca²⁺-ATPase; ER, endoplasmic reticulum.
death has been regarded as signaling checkpoints in cancer, which determine how these processes are remodeled in cancer [20]. Many studies have reported that a large influx of Ca\(^{2+}\) triggering apoptosis in cancer cells is provided by Ca\(^{2+}\) influx mediated by store-operated Ca\(^{2+}\) entry channels, which suggest a pivotal role of SOCI in apoptosis and cancer progression [21]. Moreover, the anti-apoptotic protein Bcl-2, which is commonly degraded in cancer, appears to modulate IP\(_3\)-receptor Ca\(^{2+}\) channel activity on the ER Ca\(^{2+}\) stores [10,22,23]. It was also reported that the reduction in ER means that Ca\(^{2+}\) release is insufficient to produce apoptosis [10,24]. All these results suggest that the deregulation of cellular Ca\(^{2+}\) homeostasis caused under non-physiologic condition such as irradiation can elicit cell death and determine the sensitivity of cancer cells to radiotherapy. It was also suggested that ion transports may contribute to the intrinsic radio-resistance and the survival of the tumor cell, by controlling cell cycle, metabolic adaptations or DNA repair [25]. In this study, to explore the role of cellular Ca\(^{2+}\) metabolism in sensitivity of tumor cells to radiation, the effects of gamma (\(\gamma\))-ray irradiation on cellular Ca\(^{2+}\) metabolism in radiosensitive RKO human colorectal cancer cells and A549 human lung cancer cells, one of known radiation-resistant cells, were examined.

**METHODS**

**Cell culture and Irradiation of cell cultures**

RKO human colorectal cancer cells and A549 human lung cancer cells were used. The cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured in 25 cm\(^2\) plastic tissue culture flasks at 37°C in a humidified 5% CO\(_2\)/95% air atmosphere. When the cells were in exponential growth phase at a cell density of 3x10\(^5\) cells/25 cm\(^2\) flasks, cells were irradiated with 10 Gy of \(\gamma\)-rays at a dose rate of 5.0 Gy/min with a 137 Cs irradiator (Cis biointernational IBL437C, France).

**Measurements of [Ca\(^{2+}\)]\(_i\)**

Intracellular free Ca\(^{2+}\) concentration was measured as described previously [26]. Cells were washed with PBS and incubated in 2 ml of buffer (0.05% trypsin and 0.02% EDTA). The cells were then resuspended in Tyrode solution (in mM: 140 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 1 NaH\(_2\)PO\(_4\), 5 HEPES, 5.5 Glucose and pH 7.4) and incubated at 37°C for 30 min and transferred to a recording chamber on an epifluorescence inverted microscope (Nikon Diaphot 300, Tokyo, Japan). Experimental solutions were superfused at a flow rate of 2 ml/min. Fluorescence intensity was measured using a cooled CCD camera (Photometrics PXL37, Tucson, Arizona, USA) and processed using the Axon Imaging Workbench v.2.2 (Axon Instrument, Foster city, CA, USA). [Ca\(^{2+}\)]\(_i\) was presented as the ratio of fluorescence intensities (R\(_{340/380}\)) excited by alternating illumination of 340 nm and 380 nm. Fluorescence intensity through 510 nm wavelength filter was collected using a cooled CCD digital camera (PXL-37, Photometrics, Tucson, AZ, USA). Experiments were done at 37°C.

**Solutions**

The composition of Tyrode’s solution was 140 mM NaCl, 2.5 mM CaCl\(_2\), 5 mM KCl, 1 mM MgCl\(_2\), 1 mM NaH\(_2\)PO\(_4\), 5 mM N-[2-hydroxyethyl] piperazine-N’[2-ethanesulfonic acid] (HEPES), and 5.5 mM glucose at pH 7.4. In the 0 mM Na\(^{+}\)/2.5 mM Ca\(^{2+}\) solution (Na\(^{+}\)-free solution), NaCl was isosmotically replaced by N-methyl-D-glucamine (NMDG). 140 mM Na\(^+\)/0 mM Ca\(^{2+}\) solution (Ca\(^{2+}\)-free solution) was made by omitting CaCl\(_2\). To isolate NCX activity from other Ca\(^{2+}\) pathways, 1 \(\mu\)M thapsigargin (ER Ca\(^{2+}\)-ATPase inhibitor), 5 mM caffeine (ryanodine receptor inhibitor), and 250 \(\mu\)M La\(^{3+}\) (plasma membrane Ca\(^{2+}\)-ATPase inhibitor) were added to the superfusing solutions. The 0 Ca\(^{2+}\) solution, which was used to empty the internal Ca\(^{2+}\) stores, also contained 0.1 mM EGTA and 1 \(\mu\)M thapsigargin.

**Statistical analysis**

All data were expressed as mean±SD. Statistical analysis was performed by independent t-test, with p<0.05 as criteria of significance.

**RESULTS**

**Basal level of intracellular Ca\(^{2+}\) activity**

Basal levels of intracellular Ca\(^{2+}\) activities in \(\gamma\)-ray irradiated RKO cells were compared to those in non-irradiated control cells (Table 1). When the cells were incubated for various durations up to 48 hrs after irradiation, basal levels of R\(_{340/380}\) in RKO cells were not fluctuated both in the control and irradiated cells. And no difference in R\(_{340/380}\) was observed between the control and irradiated cells. Even when the basal levels of R\(_{340/380}\) were deviated most, such as in cells incubated for 48 hrs (0.81±0.05 vs. 0.84±0.11), no statistical significance was observed (p=0.14). Based on these findings, further experiments were carried out with cells which were incubated for 48 hrs after \(\gamma\)-ray irradiation.

| Incubation time | 30 min | 1 hr | 3 hrs | 6 hrs | 12 hrs | 24 hrs | 48 hrs |
|-----------------|--------|------|-------|-------|--------|--------|--------|
| Control         | 0.80±0.05 | 0.80±0.05 | 0.81±0.05 | 0.80±0.05 | 0.81±0.05 | 0.82±0.05 | 0.81±0.05 |
| \(\gamma\)-ray irradiated | 0.82±0.05 | 0.80±0.05 | 0.81±0.05 | 0.79±0.05 | 0.83±0.11 | 0.83±0.05 | 0.84±0.11 |

Note that p=0.1379 at 48 hrs after \(\gamma\)-irradiation. n=30.
**Physiological activity of NCX**

When the cells were superfused with 0 mM Na⁺/2.5 mM Ca²⁺ solution, as described in “METHODS”, R₃₄₀/₃₈₀ in RKO cells increased to a plateau value (from 0.85±0.01 to 1.65±0.03) as shown in Fig. 1. Subsequent superfusion of 140 mM Na⁺/0 mM Ca²⁺ solution lowered R₃₄₀/₃₈₀ to the resting level with rate of R₃₄₀/₃₈₀ changes of −0.17±0.05/min (Fig. 1A and Table 2). In γ-ray irradiated RKO cells, R₃₄₀/₃₈₀ increased to a plateau value (from 0.92±0.10 to 1.66±0.20) and an additional increase to 1.87±0.40 was followed. Subsequent superfusion of 140 mM Na⁺/0 mM Ca²⁺ solution lowered R₃₄₀/₃₈₀ to the resting level with rate of R₃₄₀/₃₈₀ changes of −0.25±0.10/min (Fig. 1B and Table 2). The decay to the basal level of R₃₄₀/₃₈₀ in irradiated cells was completed faster than that of control cells (230 sec vs. 290 sec) (p<0.001).

In A549 cells, R₃₄₀/₃₈₀ increased to a plateau value (from 0.73±0.06 to 1.21±0.12). Subsequent superfusion of 140 mM Na⁺/0 mM Ca²⁺ solution lowered R₃₄₀/₃₈₀ to the resting level with rate of R₃₄₀/₃₈₀ changes of −0.13±0.06/min (Fig. 1C and Table 2). In γ-ray irradiated A549 cells, R₃₄₀/₃₈₀ increased to plateau values (from 0.73±0.06 to 1.11±0.12). Subsequent superfusion of 140 mM Na⁺/0 mM Ca²⁺ solution lowered R₃₄₀/₃₈₀ to the resting level with rate of R₃₄₀/₃₈₀ changes of −0.24±0.05/min (Fig. 1D and Table 2). The decay to the basal level of R₃₄₀/₃₈₀ was also completed faster than that of control cells (p<0.0001).

**Ca²⁺ influx via SOCl**

Ca²⁺ influx via SOCl were measured by superfusing cells with the normal Tyrode solution after emptying the internal Ca²⁺ stores. When cells were superfused with 0 Ca²⁺ solution containing 0.1 mM EGTA with 1 μM thapsigargin,
Fig. 2. Effects of γ-irradiation on SOCI in RKO and A549 cells. (A) When RKO control cells were superfused with Tyrode’s solution including 2 mM Ca²⁺ after depleting intracellular Ca²⁺ store, R340/380 was increased by Ca²⁺ influx via SOCI. (B) Ca²⁺ influx via SOCI was enhanced in γ-ray irradiated RKO cells, compared to control cells (**p < 0.0001). (C) In A549 control cells, R340/380 was increased by Ca²⁺ influx via SOCI, as in (A). (D) Ca²⁺ influx via SOCI was decreased in γ-ray irradiated A549 cells, compared to (C) (*p < 0.05). (E) Areas under SOCI response were increased by γ-ray irradiation from 213±112 to 576±304, with statistical significance (**p < 0.0001). Tracings in (A) to (D) represent the average values of R340/380.

ATP-induced Ca²⁺ release from the ER

Ca²⁺ release from the ER was measured by applying ATP extracellularly which activates IP₃ receptor channels in the ER (Fig. 3 and Table 4). When the Tyrode solution, R₃₄₀/₃₈₀ was increased from 0.77±0.06 to 1.26±0.30 (Fig. 2C). In γ-ray irradiated A549 cells, R₃₄₀/₃₈₀ increased from 0.77±0.12 to 1.10±0.24 as shown in Fig. 2D. The increments in R₃₄₀/₃₈₀ in γ-ray irradiated A549 cells were smaller than those of control cells (0.33±0.24 vs. 0.49±0.30) (p < 0.05). Areas under SOCI response were decreased by γ-ray irradiation from 221±116 to 164±95, with statistical significance (**p < 0.0001). The increment of areas under SOCI response in RKO cells by γ-ray irradiation was significantly different from that in A549 cells, which was decreased by γ-ray irradiation, as shown in Fig. 2E (p < 0.0001).
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Table 3. Comparison of SOCI-induced R340/380 changes in RKO and A549 cells

|                       | Cell type | Non-irradiated control | Gamma-irradiated |
|-----------------------|-----------|------------------------|------------------|
| **Basal level**       | RKO       | 0.76±0.09 (n=22)       | 0.74±0.09 (n=20) |
|                       | A549      | 0.77±0.06 (n=35)       | 0.77±0.12 (n=35) |
| **Peak value**        | RKO       | 1.14±0.14              | 1.60±0.44        |
|                       | A549      | 1.26±0.30              | 1.10±0.24        |
| **Changes in R340/380**| RKO      | 0.38±0.14              | 0.86±0.44***     |
|                       | A549      | 0.49±0.05              | 0.33±0.24*       |

*p < 0.05, ***p < 0.0001.

Fig. 3. Effects of γ-irradiation on ATP-induced [Ca²⁺]i changes in RKO and A549 cells. (A) Application of 100 μM ATP induced transient changes in R340/380 (ATP-induced Ca²⁺ responses) in RKO control cells. (B) In γ-ray irradiated RKO cells, transient changes in R340/380 were enhanced, compared to control cells (1.18±0.07 vs. 0.89±0.06; p < 0.0001). (C) In A549 control cells, transient changes in R340/380 were not significantly different from those in control cells. (D) In more than 60% of A549 cells measured, multiple transient changes in R340/380 were observed with a single application of ATP. (F) The frequency of multiple transient changes was increased in γ-ray irradiated A549 cells (See Table 5). Tracings in (A) to (F) represent the average values of R340/380.

ing 100 μM ATP was applied for 10 sec, R340/380 in RKO cells increased transiently, 0.83±0.05 to 1.72±0.29, and returned slowly to the basal level (Fig. 3A). In γ-ray irradiated RKO cells, R340/380 increased from 0.84±0.05 to 2.02±0.35. The difference between the peak and basal values for R340/380 in the control cells (n=24) was 0.89±0.29 and that in the γ-ray irradiated cells (n=25) was 1.18±0.35 (p < 0.0001; Table 4).

When A549 cells were superfused with the Tyrode solution containing 100 μM ATP for 10 sec, R340/380 increased transiently and returned to the basal level eliciting a single Ca²⁺ transient, as shown in Fig. 3C, in 50 cells out of 154 cells (32%) measured (Table 4). In other A549 cells, multiple Ca²⁺ transients were observed (Fig. 3E and Table 4).
Table 4. Comparison of ATP-induced R_{340/380} changes in RKO and A549 cells

| Cell type | Non-irradiated control | Gamma-irradiated |
|-----------|------------------------|------------------|
| Basal level | RKO | 0.83±0.05 (n=24) | 0.84±0.05 (n=25) |
|           | A549 | 0.75±0.11 (n=31) | 0.74±0.13 (n=18) |
| Peak value | RKO | 1.72±0.29 | 2.02±0.35 |
|           | A549 | 1.25±0.17 | 1.25±0.17 |
| Changes in R_{340/380} | RKO | 0.89±0.29*** | 0.49±0.17 |
|           | A549 | 0.49±0.17 | 0.51±0.13 |

***p<0.0001.

Table 5. Changes in R_{340/380} induced by ATP in A549 cells

| Events with 1 peak | Control (n=154) | γ -ray (48 hr) (n=167) |
|--------------------|-----------------|----------------------|
| Number of event    | 50              | 19                   |
| Basal level        | 0.75±0.14       | 0.74±0.10            |
| Peak value         | 1.25±0.21       | 1.25±0.13            |
| Changes in R_{340/380} | 0.49±0.21 | 0.51±0.10            |
| 2 peaks            |                 |                      |
| Number of event    | 44              | 28                   |
| Basal level        | 0.73±0.07       | 0.66±0.05            |
| Peak value         | 1.41±0.20       | 1.28±0.16            |
| Changes in R_{340/380} | 0.68±0.20 | 0.63±0.16            |
| 3 peaks            |                 |                      |
| Number of event    | 32              | 30                   |
| Basal level        | 0.73±0.06       | 0.67±0.05            |
| Peak value         | 1.48±0.17       | 1.31±0.22            |
| Changes in R_{340/380} | 0.75±0.17 | 0.64±0.22            |
| Multi peaks        |                 |                      |
| Number of event    | 28              | 90                   |
| Basal level        | 0.71±0.05       | 0.69±0.09            |
| Peak value         | 1.41±0.16       | 1.33±0.47            |
| Changes in R_{340/380} | 0.69±0.16 | 0.65±0.38            |

The amplitudes of Ca^{2+} transients, the differences between the peak and basal values of R_{340/380}, were not significantly different between control cells and γ -ray irradiated cells (Table 4). However, the frequency of multiple transients was increased in γ -ray irradiated cells (Table 5).

DISCUSSION

Surge of intracellular Ca^{2+} causing cell death can arise from a variety of sources. [Ca^{2+}]_{i} can be increased by the entry of extracellular Ca^{2+} via SOCI or the release of stored Ca^{2+} from the ER [14-17]. Ca^{2+} influx via reverse mode of NCX also contribute to [Ca^{2+}]_{i} increase [17,27,28].

By γ -irradiation, RKO cells begin to exit from G2/M arrest to apoptosis by 24 hrs after irradiation. Only small fractions of cells remain in G2/M phase by 48 hrs, implying that the post-mitotic apoptosis occurs by 48 hrs after irradiation [29]. During this time span, basal level of [Ca^{2+}]_{i} in RKO cells remained relatively unchanged (Table 1) although irradiation elicited enlargement of viable cells (data not shown). Thus experiments were done with cells incubated for 48 hrs after γ -ray irradiation.

The change in [Ca^{2+}]_{i}, via reverse mode of NCX can be measured by blocking other cellular Ca^{2+} pathways as previously reported [26,27]. Irradiation does not seem to influence NCX ability to import Ca^{2+} into the cytosol of both RKO and A549 cells (Fig. 1). The forward mode of NCX plays a major role in clearing Ca^{2+} out of cytosol and can be measured by the decline of [Ca^{2+}]_{i}, as shown in Fig. 1. Interestingly, irradiation tends to speed up the pumping activity of NCX forward mode in both cells (Table 2). It is not clear that irradiation-induced pumping activity has any physiological role in cellular metabolism. Meanwhile, to understand the cause for additional increase in [Ca^{2+}]_{i}, via NCX over the plateau region, more information on the irradiation-induced changes in membrane fluidity is needed, since an enlargement of cells by irradiation was observed (data not shown).

Depletion or depression of Ca^{2+} content from ER can signal long-term cellular responses such as gene expression and programmed cell death or apoptosis [30,31] and provides a signal that activates Ca^{2+} entry through the SOCI channels [32,33]. Enhancement of Ca^{2+} entry via SOCI in RKO cells by irradiation, as shown in Fig. 2, may contribute to promotion of cell death. The enhancement of the SOCI activity may be a consequence of other cellular changes induced by irradiation, such as emptying of the ER following the increased Ca^{2+} release by irradiation (Fig. 3B). Irradiation may induce direct effects on SOCI-modulating proteins such as STIM and synergistic interaction of SOCI with other cellular components as reported in studies of irradiation-induced BAX interaction with SOCI [34,35].

The data of Fig. 2 provide indirect information on the ER content of Ca^{2+}. Pre-emptying the ER to induce Ca^{2+} influx via SOCI can estimate the size of releasable Ca^{2+} pool. The results of Fig. 2 and Table 3 imply that the Ca^{2+} content in the ER of RKO cells is much greater than that of A549 cells. The amounts of Ca^{2+} released from the ER
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by ATP also feature the same character: RKO cells release greater amount of Ca\(^{2+}\) than A549 cells do (Table 4).

Interestingly, \(\gamma\)-irradiation on A549 cells elicited decrements of ER Ca\(^{2+}\) content and Ca\(^{2+}\) influx via SOCI, while \(\gamma\)-irradiation on RKO cells resulted in enhancements of Ca\(^{2+}\) influx via SOCI (Fig. 2). These results, along with enhanced Ca\(^{2+}\) release from the ER by ATP in RKO cells as shown in Fig. 5, can provide possible explanation for distinct difference in cell death between RKO and A549 cells. Assuming that Ca\(^{2+}\) flux from the ER promotes cell death [20], enhanced Ca\(^{2+}\) release from the ER in RKO cells by \(\gamma\)-irradiation may explain radio-sensitivity of RKO cells. Unchanged Ca\(^{2+}\) release from the ER may be one of possible mechanisms for radiation resistivity of A549 cells. These observations are well supported by other reports stating that Ca\(^{2+}\) released from the reduction in ER is not sufficient to produce apoptosis [10,24].

Not surprisingly, it was found that the activity of Ca\(^{2+}\) transporters of A549 cells investigated in this study was not as much affected by \(\gamma\)-irradiation as that of radio-sensitive RKO cells. However, \(\gamma\)-irradiation increased the incidence of multiple Ca\(^{2+}\) peaks in A549 cells which suggests that Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism was activated by \(\gamma\)-irradiation (Table 5). To clarify the involvement of this Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism in radiation-induced Ca\(^{2+}\) deregulation, further study with immunochromatis and molecular biological methods will be needed [36]. However, the resting values of [Ca\(^{2+}\)]\(_{i}\) were not increased by multiple Ca\(^{2+}\) transients (Table 4). The results of Table 2 and 3 also support the theme that \(\gamma\)-irradiation does not affect intracellular Ca\(^{2+}\) metabolism of A549 cells and these cells may not employ the Ca\(^{2+}\)-activated cellular process of cell death. In conclusion, these results suggest that \(\gamma\)-irradiation enhances the cellular Ca\(^{2+}\) metabolism in radiation-sensitive RKO cells and elicits programmed cell death. The results of this study may provide further understanding of the role of Ca\(^{2+}\) in promoting cell death and the opportunities for therapeutic intervention of cancer.

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