Real-time cell-impedance sensing assay as an alternative to clonogenic assay in evaluating cancer radiotherapy

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Abstract Intrinsic radiosensitivity of normal and tumour tissues has been shown to be an independent prognostic factor for patients' response to radiotherapy. This study compares the real-time cell-impedance sensing (RT-CES) assay with the conventional clonogenic assay in terms of in-vitro radiosensitivity. One objective in this study was to predict in-vivo response to gold nanoparticle (GNP) treatment on the basis of in-vitro RT-CES testing results. Four adenocarcinoma cancer cell lines were tested using both the RT-CES and the clonogenic assays. Cell-survival curves were plotted, and the mean SF2 values obtained by these two different assay methods were compared using ANOVA. Radiation sensitivities obtained in-vitro were then compared with the in-vivo results. On the basis of the measurement of cell colonies, the RT-CES assay has similar radiosensitivity to the clonogenic assay, but significantly shortens the testing time from 14–21 days to only 72 h. Intrinsic GNP enhanced radiation sensitivity using tumour volume (mm$^3$) in vivo is comparable with that using RT-CES cell survival assay in vitro. Furthermore, the RT-CES system provides real-time information regarding the state of cell radiosensitivity that may give useful information towards personalizing radiotherapy. The RT-CES assay enables more reliable and time-efficient results in the evaluation of radiosensitivity.

Keywords Cancer radiotherapy · Radiosensitivity · Clonogenic assay · RT-CES cell-proliferation assay

Introduction

Radiosensitivity has been considered one of the most important radiobiological factors in determining how tumours respond to radiotherapy. Using the clonogenic assay to determine the cell surviving fraction at 2 Gy (SF$_2$) and the intrinsic radiosensitivity has been shown to be an independent prognostic factor governing how patients respond to radiotherapy in carcinoma of the cervix [1] and other types of cancers [2, 3]. Gold nanoparticles (GNPs) have been found to enhance tumour cells’ sensitivity to irradiation, including in prostate and breast cancer [4, 5]. With ortho-voltage irradiation, glucose coated GNPs (Glu-GNPs) showed significant enhancement in tumour growth inhibition compared with X-ray treatment alone [6]. GNPs induced G2/M arrest is one of the important mechanisms enabling the GNPs to increase tumour cells’ sensitivity to radiotherapy [6].

Radiotherapy can damage DNA and also lead to accumulation of damage in cancer cells, causing them to die or
reproduce more slowly. Therefore, measuring cell proliferation ability is an important indication in evaluating the cancer’s sensitivity to radiotherapy [7]. The clonogenic cell-survival assays were initially used to study the effects of radiation on cells and have played an essential role in radiobiology [8, 9]. A cell-survival curve defines a dose relationship between radiation used to kill targeted cells and inhibit the cells’ ability to reproduce. These assays are also widely used to examine the effects of radiotherapy alone and in conjunction with chemotherapy or gene therapy. Survival curves have been generated for many established cultured cell lines.

Although the clonogenic assay is generally considered an optimum test system for in-vitro radiation studies, the problems inherent with the clonogenic assay are also well known [10, 11]. These problems include a low assay success rate, technical difficulties of the assay which limit clinical usefulness, and the long time (2–4 weeks) required to generate a result. A number of attempts have been developed to replace the clonogenic assay [12–16], but these methods have mostly depended on colorimetry to measure cell viability, yielding data which may not relate to cell proliferation. As a result, overestimation of cell survival in these assays might result when the control (non-irradiated cells) reaches confluence, or the delayed expression of radiation damage cannot be identified because of the short experimental duration [17].

To overcome these limitations, in this study the real-time cell electronic sensor (RT-CES) cell-proliferation assay was developed to evaluate GNP enhanced radiotherapy sensitivities. RT-CES assay has proven valuable and reliable for real-time monitoring of dynamic cell changes, including cell proliferation and changes induced by cell–toxicant interaction [18, 19]. The basic principle of the RT-CES system is to monitor the changes in electrode impedance induced by the interactions between test cells and electrodes [18, 19]. The more cells attached to the sensor, the higher the impedance that could be picked up by RT-CES. Therefore, the dynamic data generated by the RT-CES can truly reflect cell proliferation. The RT-CES dynamic model has shown great potential in capturing cell dynamics in the presence of toxicants, and in predicting cell response [20–22].

Materials and methods

Synthesis of thio-glucose-capped gold nanoparticles

The general synthetic method for making gold nanoparticles has been described [4]:

1. 3.2 mL 25 mmol L\(^{-1}\) HAuCl\(_4\) solution was added to 60 mL of deionized water in an ice bath with moderate stirring

2. the naked GNP solution was added into tube containing 4 mL 20 mmol L\(^{-1}\) 1-thio-β-glucose.

Thio-glucose covalently bound to the GNPs to form functionalized thio-glucose-capped gold nanoparticles (Glu-GNPs). The average size of Glu-GNPs as measured by dynamic light scattering (DLS) was approximately 10.8 nm.

Cell culture

The four most commonly used human adenocarcinoma lines, prostate carcinoma DU-145 line, breast cancer MCF-7 line, lung cancer A549 line, and H460 line, were used in all of the experiments. All cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 20 mmol L\(^{-1}\) d-glucose, 100 UI mL\(^{-1}\) penicillin G, and 100 μg mL\(^{-1}\) streptomycin in a humidified incubator with 5% CO\(_2\), at 37 °C. DMEM without glucose was used for the cells that were exposed to Glu-GNPs.

Tumour-bearing mouse model

The experimental animal procedures were approved by the institutional animal care committee. MCF-7 cells were diluted with the culture medium without serum or antibiotics to a concentration of 5×10\(^6\) cells per 0.2 mL.

Four to five-week-old BALB/c mice were used in this experiment. The mice were maintained in accordance with the institutional committee approved guidelines. Each was inoculated with 5×10\(^6\) cells in the right flank. The tumour was allowed to grow for 10 days. The tumour sizes were determined on the basis of the standard method as described in Ref. [23], and thus the tumour model was successfully constructed.

Irradiation

In-vitro irradiation for cells

All cell-irradiation treatments were carried out using a Pautak Therapax 3 Orthovoltage (244 monitor-units min\(^{-1}\)) X-ray machine at 200 kVp using a 0.35 CU + 1.5 AL filter. The targeted cells grew in 100-mm culture dishes and reached approximately 75% confluence. After treatment of the cells with or without Glu-GNPs for two hours, cells in the control group received a mock treatment while experimental cells were irradiated with 1, 2, 4, 6, 8, or 10-Gy doses. After irradiation, the cells were collected for either clonogenic or RT-CES cell-survival assays.
In-vivo irradiation for tumour tissue or tumour-bearing mice

The tumour-bearing mice were randomly separated into five groups.

(A) The first group was for in-vitro study. Primary cancer cells were isolated from tumour tissue of the mice, on the basis of a previously reported procedure [24, 25], to be cultured in DMEM for 24 h, after which the cells were ready for experiments. After treatment with X-rays alone, Glu-GNPs alone, or a combination of X-ray and Glu-GNPs, 5,000 cells in 150 μL were seeded on to the RT-CES chip plate and cell survival was measured by the RT-CES assay.

(B) The other four tumour-bearing groups (n=4) were studied in-vivo: control (tumour growth without treatment), Glu-GNPs alone, radiotherapy alone, and radiotherapy+Glu-GNPs. The mice underwent tail-vein intravenous injection of GNP fluid. Glu-GNPs were injected (i.v.) at 3.3 mg μL⁻¹ in 100 μL normal saline weekly for four weeks. Two hours after each Glu-GNPs injection the mice were treated with 200 KVP X-rays at a dose 10 Gy. Tumour volumes, normalized to day 1, were compared 10 days after the 4-week treatments. To compare tumour response in nude mice, the product of the diagonal width and length of the tumour was taken as a measure of tumour size.

Clonogenic cell-survival assay

Clonogenic survival assay was performed on the basis of a standard procedure with slight modification [8]. A concentration of 25×10³ cells with or without treatment was seeded into 6-cm culture dishes in 5 mL growth medium. Dishes were incubated to allow colony formation for 10–14 days. The colonies were first fixed with formaldehyde (4%) for 2 h and then stained with Trypan blue for 30 min. The colonies were counted with the free software ImageJ (NIH Image) described by Niyazi et al. and Dahle et al. [26, 27]. Each assay was made in triplicate and only colonies containing at least 50 cells were counted. Radiation survival curves were constructed by normalizing the number of surviving colonies after jointly treating the number of surviving colonies with irradiation alone or in combination with Glu-GNPs.

RT-CES cell-survival assay

The detailed description of the RT-CES system (ACEA Biosciences, San Diego, CA, USA) has been reported elsewhere [18, 19]. Cells attached to the electrode sensor arrays result in an impedance change. Generally speaking, the more cells attached to the sensor array, the larger the impedance [18, 19]. Changes in impedance corresponding to individual microwells were recorded in parallel, the signals were then collected by the computer, and all impedance data were automatically converted to the cell index (CI) using a built-in calibration (Fig. 1).

For the RT-CES cell-survival assay, after treatment with irradiation alone or in combination with Glu-GNPs, the cancer cells with the initial population of 300 cells (14 days test) or 1,500 cells (for 72 h test) in 150 μL volume were seeded separately into individual microwells. The control cells without treatment on the same E-plate were maintained in parallel. The cultures were continuously monitored for 14 days. The CI data were used to calculate dose response curves.

Statistical analysis

1. Calculation of coefficients of variation (CV), where CV=standard deviation (SD)/mean, was used as a quantitative indicator of the repeatability of the tests.
2. One-way ANOVA was used to compare the surviving fraction at 2 Gy (SF2) of the cell lines using two different assay methods. After confirmation using the experimental results in Ref. [28], the variances were found to be equal. The Bonferroni pairwise multiple comparison test [29] was also used if the difference in mean SF2 values were significant.
3. The correlation of the radiation sensitivity between survival rate measured by the RT-CES and the inhibitive rate of normalized tumour volume was evaluated using the Student’s t test, with the differences at a P value of less than 0.05 considered to be statistically significant.

Results

Measurement of cell proliferation

The cell proliferation is embodied in increased cell numbers in RT-CES assay or colony numbers in clonogenic assay. Therefore, the CI value of cell proliferation obtained using the RT-CES system should be quantitatively correlated with the colony numbers. MCF-7 cells were titrated and grown on the sensing devices, and were then accurately measured by the RT-CES system. The results in Fig. 2a show that the RT-CES system was able to monitor the dynamic proliferation of MCF-7 cancer cells with their characteristic growth patterns. The CI values measured by the RT-CES system at 48 h (Fig. 2a) were linearly correlated (R²=0.958) with the colony numbers over a range of 9.5 to 226 colonies determined by clonogenic cell survival assay on the 14th day (Fig. 2b). To evaluate the accuracy of the RT-CES
assay, the CI values for each colony number were measured by three replicates with appropriate cell numbers. CV ranged from 3.2% for high cell colony numbers to 8.7% for low cell colony numbers. Cell titration was also assessed and there were similar correlation coefficients for both clonogenic cell survival assay with 0.9897 and RT-CES test with 0.9782. The results indicate that efficiencies achieved by the RT-CES system were similar to the clonogenic cell survival assay for measuring cell survival.

Radiation dose response

MCF-7 cells were treated with 200-kVp X-rays with doses of 1, 2, 4, 6, 8, and 10 Gy, then tested for cytotoxicity response using the RT-CES system and the clonogenic assay. The results in Fig. 3a show that the RT-CES assay was able to monitor the dynamic proliferation of MCF-7 cancer cells treated by X-rays. Although the control cells were not irradiated, they showed a similar lag as those irradiated in the first 30 h, because the starting cell number in this experiment was small. After 40 h, only a negligible difference existed between the cellular inhibitions of samples irradiated at different doses. Subsequent measurements showed that the RT-CES assay differentiated dynamic cellular curves resulting from various X-ray doses (Fig. 3a). The dose responses of CI values determined on the RT-CES system at 72 h were linearly correlated ($R^2=0.973$) with the colony numbers.

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**Fig. 1** The mechanism of the RT-CES cell-survival assay

**Fig. 2** Correlation of MCF-7 cell proliferation as measured by the cell index using RT-CES and colony number obtained with the clonogenic assay. (A) Dynamic change of MCF-7 cell proliferation. (B) Correlation between cell index values from the RT-CES assay at 48 h and colony numbers from the clonogenic assay at 14 days
determined by clonogenic cell survival assay on the 14th day (Fig. 3b).

We treated MCF-7, A549, H460, and DU145 cancer cells with different X-ray doses and tested cytotoxicity responses using the RT-CES system and clonogenic assay. The SF2 values obtained from both assays are summarized in Table 1. After a specific time interval had passed after irradiation (72 h for RT-CES assay and the 14th day for clonogenic assay) the SF2 values obtained from different cell types between the RT-CES system and the clonogenic assay were comparable, indicating that the RT-CES system is a reliable, label-free tool that can be used to replace clonogenic assay to assess radiotherapy sensitivity. According to the RT-CES testing results, the SF2 ranged from 0.32 to 0.66. The most radiosensitive cell line is the MCF-7 cell whereas H460 was the most radioresistant. The radiation sensitivity is correlated to the growth rate of each cell line. Dynamic changes of the relative growth rates of cell lines (induced by 2 Gy irradiation) can be calculated on the basis of real-time cell-index monitoring, as shown in Fig. 4. $P > 0.05$ for all cell lines (Table 1) indicates that at the 0.05 significance level there is not sufficient evidence to show the existence of a difference between SF2 values generated by the RT-CES assay and those generated by the clonogenic assay.

**Nanoparticles enhanced radiotherapy in vitro**

The RT-CES survival assay was also used to determine whether Glu-GNPs enhanced radiation sensitivity. Proliferation of the cells was dynamically monitored using the RT-CES assay. The responses of the MCF-7 cell line with and without either GNPs or X-ray treatment are shown in Fig. 5a. After irradiation, 200-kVp X-rays (10 Gy) induced a significant decrease in cell survival (Fig. 5b). However, 32.5% of cancer cells still survived after 72 h and 21.3% of cells survived after 120 h. For the cells exposed to Glu-GNPs (15 nmol L$^{-1}$), irradiation with 200-kVp X-rays (10 Gy) resulted in 8.8% of cells surviving after 72 h and complete cancer-cell death after 120 h (in both RT-CES and clonogenic assays). Significant differences were obtained when comparing survival rates between radio-

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Mean SF2 (RT-CES assay)</th>
<th>SD</th>
<th>Mean SF2 (clonogenic assay)</th>
<th>SD</th>
<th>ANOVA$^a$ (p)</th>
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<tr>
<td>MCF-7</td>
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<td>0.31</td>
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<tr>
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<td>0.01</td>
<td>0.52</td>
<td>0.02</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>H460</td>
<td>0.66</td>
<td>0.02</td>
<td>0.63</td>
<td>0.01</td>
<td>$P &gt; 0.05$</td>
</tr>
</tbody>
</table>

$^a$ One-way analysis of variance. Multiple comparison test was used to test if the difference between mean SF2 values were significant.
therapy alone and Glu-GNPs plus radiotherapy at 72 h and 120 h ($P<0.05$).

Correlation of in-vitro responses in the RT-CES assay with in-vivo behaviour in mice

To determine if the RT-CES cell-survival assay can be used to predict radiosensitivity, the breast cancer-bearing mice were randomly divided between one cell treatment group for in-vitro study and four other tumour treatment groups for in-vivo study. In the first group, the cancer cells were isolated from tumour tissue of tumour bearing mice, then treated with X-rays and Glu-GNPs either alone or in combination in-vitro. After treatment, the cells were seeded on to the RT-CES chip plate and cell survival was measured by the RT-CES assay. Glu-GNPs (15 nmol L$^{-1}$) caused 7.37% inhibition of the cancer tumour cells. At an X-ray dose of 10 Gy, inhibition of cell proliferation was observed to be approximately 37.72%. However, when the tumour cells were treated with GNPs plus X-rays, approximately 60.72% inhibition was obtained (Fig. 5a).

For the in-vivo study, the tumour volumes were used as markers. The concentration of gold in cancer tissue was 0.095 μg g$^{-1}$ tissue (15 nmol L$^{-1}$) 2 h after injection of Glu-GNPs. Glu-GNPs, X-rays, and both in combination induced 8.56%, 35.48%, and 58.45% tumour shrinkage, respectively. The results in both the in-vitro and in-vivo studies indicated a much better tumour control effect with radiotherapy plus the Glu-GNPs than radiotherapy alone. A relationship between the percentage decrease in CI value and the tumour volume in-vivo response can be demonstrated (compared with X-rays alone, enhancement was 36.92% for in vitro (Fig. 6a) and 35.61% for in vivo (Fig. 6b)). The degree of inhibition of cellular proliferation determined by the RT-CES assay can be used to predict tumour suppression by radiotherapy.

When comparing in-vitro and in-vivo results with student’s $t$ test, $P$ values of less than 0.05 were obtained for Glu-GNP alone, radiotherapy alone, and the combination of Glu-GNP and radiotherapy. These results indicate that RT-CES assay using primary cancer tissue cells can be used to predict the inhibitive rate of normalized tumour volume in tumour-bearing mice.

Discussion

Although several methods, including MTT assay [14] and sulforhodamine B assay [15], have been developed for radiosensitivity testing, measurement of cell proliferation is a more accurate method for evaluating cytotoxicity resulting from radiation therapy. Therefore, clonogenic assays were considered to be the “gold-standard assay” in radiobiology [25]. However, a number of drawbacks in current available clonogenic assay techniques [30, 31] have restricted these methods for routine applications for many years. First, the assay is very time-consuming. Irradiation damages the cells’ DNA, creating defects which propagate...
through cell division. To observe these defects, the assay duration should be adjusted to double that of the cell growth time. Clonogenic assay can only observe cell colony formation after at least 4–6 cell divisions. Therefore, the test is very time consuming and requires a 2–4 week testing period depending on cell type. Second, the 2–4 weeks-long culture period creates difficulties in the maintenance of the clonogenic assay resulting in lower assay success rate. Finally, because of long culture time, the control cells have to remain in exponential growth up to the end of the assay.

Although the RT-CES assay has been reported to have potential for measurement of the effect of radiation on cancer cell proliferation [32], methodological details, assay validity, and interpretation of data in contrast with clonogenic assays for evaluating radio-sensitivity needs further investigation. In this study, the data generated by the RT-CES system were well correlated with those generated from the clonogenic assay (Fig. 2b). A linear relationship with $R^2=0.958$ between the RT-CES assay and the clonogenic assay indicates that the RT-CES cell proliferation assay can replace the clonogenic assay to evaluate cell proliferation.

The RT-CES assay also fulfilled the requirements of the radiotherapy sensitivity assay in this study. A linear dose–response relationship with $R^2=0.965$ is shown in Fig. 3a. The fact that the RT-CES cell-survival assay with cell index yielded regression equations (Fig. 3b) at 72 h that were very close to those produced by the clonogenic assay with cell colony count ($R^2=0.974$) on the 14th day indicates that the RT-CES assay can be used for estimating radiotherapy sensitivities.

The SF$_2$ values generated by either the RT-CES or the clonogenic assays for four cancer cell lines were compared. Based on the SF$_2$ values of X-rays in three validation tests, the RT-CES assay provided good intra-laboratory reproducibility with CV values ranging from 0.018 to 0.064 (Table 1) and supported by the ANOVA analysis. On the basis of the SF$_2$ values, the RT-CES system achieved a sensitivity level in 72 h equal to that in the clonogenic assay on the 14th day. For the four different cell lines tested, the RT-CES assay was a viable alternative test system for in-vitro radio-sensitivity tests. Within the relevant dose ranges, it clearly produced comparable outputs to the “gold-standard” clonogenic assay [11, 12]. One disadvantage of clonogenic assay is its tendency to overestimate radiotherapy cytotoxicity.

The results of this study also support the conclusion that the RT-CES cell proliferation in-vitro assay can be used to predict radiation sensitivities in-vivo. When breast cancer cells isolated from breast cancer tumour-bearing mice were treated with X-rays, the changes in cell proliferation measured by the RT-CES assay in-vitro corresponded to the tumour shrinkage in-vivo. This indicates that results obtained using the RT-CES assay are similar to those using the clonogenic assay and thus the RT-CES assay can be used to predict the radiotherapy sensitivity for cells with known growth properties over 72 h. However, a longer incubation period should be used for cells that have a longer doubling time, especially if it is applied to primary cultures that require much longer dividing periods than cultured cell lines. A longer incubation period may also be needed for other cases, e.g., radiation-induced cell-cycle arrest. At a sufficiently high dose of radiation, some cells will cease dividing after undergoing a few growth cycles.

The combination of radiotherapy sensitizer (GNPs in this case) and radiotherapy is a common strategy to treat many advanced cancers, including breast cancer and other types [33]. We have analyzed the effects and dynamic behaviour of a combined GNP plus ionizing radiation regimen on cytotoxicity and apoptosis in a breast cancer tumour animal model. The experimental results show that dynamic behaviour measured by the RT-CES assay in-vitro matches the tumour volume shrinkage in-vivo. The RT-CES assay is well suited to investigating enhanced radiotherapy induced by a radiation sensitizer. The dynamic behaviour of interactions measured by the RT-CES assay can also provide useful data to study cancer killing mechanisms [6].
to optimize radiotherapy administration. Ultimately, the RT-CES assay can be extended to study a wide range of sensitizers for either radiotherapy or chemotherapy.

One unique advantage of RT-CES technology is the incorporation of dynamic properties into a cell-based platform useful in establishing a computer model system to predict toxicities [20, 29]. The possibility of using RT-CES dynamic data to establish a computer model system for predicting radiotherapy sensitivity in cancer will be explored in future investigations.

In conclusion, we have performed in-house assessments of the RT-CES cell proliferation assay for studying radiation sensitivity in a conventional laboratory setting. The results obtained by the RT-CES assay agree with the data generated by the clonogenic assay. The RT-CES assays were simple to operate and provide sensitive measurements comparable with the clonogenic assay. The RT-CES assay also significantly shortens testing time from 14 days required by the clonogenic assay to only 72 h. More importantly, the assay can be used to test radiation sensitivity of primary cells in-vitro, providing useful data for us to predict the cancer sensitivity to radiation therapy in-vivo.

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References


