Growth Fraction and Cell Cycle Time of Gamma-rays Irradiated Mammalian Cells

SHUNSAKU Sasaki

Department of Radiation Research, Tohoku University School of Medicine, Sendai

Sasaki, S. Growth Fraction and Cell Cycle Time of Gamma-rays Irradiated Mammalian Cells. Tohoku J. exp. Med., 1972, 108 (3), 225-237 — Population kinetic analysis was made for growth fraction and median cell cycle time over 9 days after irradiation of a mouse mammary tumor cell population (FM3A) treated with 500 and 700 R of 60Co gamma-rays, where the growth fraction was estimated from labeling indices determined by the staggered addition method with 3H-thymidine and colchicine, and the median cell cycle time from the rate of c-mitosis accumulation by colchicine as well as the growth fraction. The analysis revealed a modest proliferation of the median cell cycle time of proliferating cells in the gamma-irradiated population. Irrespective of the doses of irradiation, the most remarkable reduction in the growth fraction was noted to occur on the third day of irradiation. The data obtained indicate that the diminution of the rate of increase in total cell number is derived primarily from decrement of the growth fraction and not largely attributable to prolonged cell cycle time, and the decline in the growth fraction is due to departure from the cell cycle and/or death of the cell.

irradiation; cell cycle; growth fraction

There are numerous circumstantial evidences that the main target of radiation causing cellular death lies in the nucleic acids, especially DNA. A considerable amount of studies have been reported on detectable changes which occur in DNA immediately after irradiation, e.g. strand scission, and it appears that most of these deteriorating effects are rather transitory and followed by a prompt repair. However, our present knowledge is too scanty to elucidate the immediate cause of death of the cell and the mechanism of molecular events whereby lethal damages caused by irradiation and born by the cell manifest themselves.

If the doses of radiation are not extremely high, radiation-induced sterile cells go through several cell cycles and eventually they die (Elkind et al. 1963, Sasaki 1969). This type of radiation-induced death of the cell is usually called a reproductive death. This investigation was planned to obtain basic data to facilitate the dynamic understanding of the delayed expression of lethal damage brought about by radiation.

Reports on kinetic behaviors of irradiated cells are not a few. For example, mitotic delay and suppression of DNA synthesis after irradiation have been investigated in detail (Elkind and Whitmore 1967). However, these effects

Received for publication, March 31, 1972.
represent only small parts of response of the irradiated cells, and their relations to cellular death are obscure. Watanabe and Okada (1966) conducted a population kinetic study in an attempt to determine the stage in the cell cycle at which radiation-induced death of the cell took place, and concluded that such a cellular death occurs in the G1-stage of the cell cycle. The lapse-time cinemagraphic studies of irradiated cells conducted mainly by pedigree analysis have supplied an accurate information on the behaviors of individual cells (Froese 1966, Marin and Bender 1966, Thompson and Suit 1967, Hurwitz and Tolmach 1969), but these observations are limited to only several generations after irradiation.

The present communication describes the results of consecutive measurements of the growth fraction and of the median cell cycle time during a fairly long period after irradiation. Methods which facilitate determination of the growth fraction and cell cycle time as quickly as possible are desirable for an analysis of an irradiated cell population, and considerable difficulties are inherent in such analysis when attempted by the conventional methods. In view of this a few newly devised methods will be introduced first. Both the growth fraction and the median cell cycle time were determined by these methods for a murine mammary tumor cell population (FM3A) irradiated with 500 and 700 R of 60Co gamma-rays.

**MATERIALS AND METHODS**

1) **FM3A cells**

FM3A cells were derived from a cell line originating from spontaneous mammary tumor cells in a C3H mouse, established by Nakano (1966) as suspension culture in vitro. The culture medium was Eagle's minimum essential medium containing 10 per cent calf serum and added with sodium pyruvate (1.0 mM) and L-serine (0.1 mM). Cell population densities were determined by means of a calibrated Neubauer's hemocytometer. The macroscopic colony forming ability was estimated by the soft agar method (Sato et al. 1967). The plating efficiency of non-irradiated cells ranged from 90 to 95 per cent thereby.

2) **60Co gamma-rays irradiation**

Cell suspensions at concentrations from 1.0 to 3.0 × 10^5 cells per ml in culture bottles were irradiated at room temperature with 500 or 700 R of 60Co gamma-rays at a dose rate of 23.5 R/min. The cultures were returned to an incubator at 37°C within 5 minutes upon conclusion of irradiation.

3) **Preparation of smears for determination of mitotic index**

The technique of Puck and Steffen (1963), with a slight modification, was employed. Namely, cells collected by centrifugation (800 to 1,000 rpm, 3 min) were resuspended in ZmM phosphate buffer (pH 7.2) containing 0.30 per cent sodium chloride, and the resulting suspension was allowed to stand for five minutes. The suspension was centrifuged at the same rate for the same length of time, and the cells were fixed in an ethanol–acetic acid (3:1) mixture for five minutes, followed by addition of two parts of 65 per cent acetic acid to one part of fixed cell suspension. After the subsequent storage for overnight at −15°C, smears were made with these cells, and the slides thus prepared were treated with 1.0 N HCl at 60°C for 6 min, rinsed with water and stained with crystal violet.
Proliferation Kinetics of Irradiated Cells

4) Microautoradiography

Smears of cells containing \(^3\)H-labeled DNA were treated in ice-cold 2 per cent triacetate acid solution for two minutes, rinsed with water, and allowed to dry in air. They were then dipped in Sakura NR-M2 liquid emulsion (Konishiroku Photo Ind. Co., Ltd.) and exposed for ten days, followed by development and staining with crystal violet.

5) Trypan-blue staining

In order to know the proportion of dead cells in a cell population, the viability of cells was measured from the cellular ability to exclude trypan-blue (Eaton et al. 1959). That is, one part of a 1.0% trypan blue solution was added to two parts of a cell suspension, and the dead cell fraction was determined by making microscopic counts of more than 1,000 treated cells within 30 minutes.

Method for Estimation of the Growth Fraction

The growth fraction has been defined by Mendelsohn (1962) as the proportion of "the growing cells" in a cell population. In his method, the growth fraction is calculated from labeling indices of a cell population 5 days after pulse labeling with \(^3\)H-thymidine, whereupon labeled cells are regarded as 'the growing cells'. A disadvantage inherent in this method lies in the fact that a fairly long period of incubation for as many as five days is required for determination of the growth fraction. It is impracticable to make accurate estimation of the growth fraction, if a transition from 'the growing cells' to 'the non-growing cells', that is, cell death or cell loss occurs during the incubation period. Inasmuch as such events undoubtedly take place in a cell population that has been irradiated with ionizing radiation, the growth fraction needs to be determined in a short period of time.

The method of Lala and Patt (1966) is also based on the assumption that cells capable of DNA synthesis are regarded as 'cycling cells'. Namely, the growth fraction is estimated from the times of each phase of the cell cycle that has been estimated by the labeled mitosis method and from the labeling index determined by the pulse label. This method facilitates an estimation of the growth fraction in a shorter length of time than that by the method of Mendelsohn. However, it is also practicable to measure the proportion of cells capable of DNA synthesis in a cell population by a more direct method as described below.

We assessed applicability of a method where the growth fraction is estimated by the time-different addition of \(^3\)H-thymidine and colchicine. When \(^3\)H-thymidine is added, cells at the DNA synthetic phase (S-phase) become promptly labeled and further incubation leads to labeling of cells which have been at the G1-phase. If colchicine is added after a lapse of time equal in length to that of G2-phase (T(G2)), the cells in the culture fail thereafter to divide. This method must facilitate labeling of all cells capable of DNA synthesis. The labeling index must remain constant once all the cells, which were in the G1-phase and G2-phase at the time of \(^3\)H-thymidine addition, have reached the subsequent S-phase. Therefore, this labeling index must be eventually equal to the growth fraction in a cell population at the time of colchicine addition.

As irradiated cells had an average T(G2) of about 2 hours which was noted to be almost equal to that exhibited by non-irradiated cells by the labeled mitosis method, colchicine was added to the culture at the end of 2 hours after addition of \(^3\)H-thymidine.

Fig. 1 illustrates, as an example, the process of estimation of the growth fraction by this method on the 3rd day's cell population after irradiation with 700R. Results of a simple continuous labeling with \(^3\)H-thymidine alone are also presented for comparison. As can be noted, labeling indices were almost constant between 5 hours and 9 hours after addition of \(^3\)H-thymidine as assayed by the time-different addition method. It was observed that the indices increased progressively with the lapse of time of incubation when assayed in the simple continuous labeling. As assays with the former system showed an average labeling index of 0.524 during the plateau phase, this value was considered to represent the growth fraction.
Fig. 1. Estimation of the growth fraction of a cell population at the 3rd day of irradiation with 700 R. Labeling indices were determined by adding $^3$H-thymidine at 69 hr and colchicine at 72 hr after irradiation. Labeling indices for plateau phase averaged 0.524 which must be equal to the growth fraction.

To determine a labeling index, at least one thousand cells were examined. A cell with ten grains or more on the nucleus, which were examined by microautoradiography, was regarded as positive. Final concentration of $^3$H-thymidine was 0.2 $\mu$Ci/ml (200 $\mu$Ci/mmole), and that of colchicine was 1.0 $\mu$g/ml.

**Method for Estimation of Cell Cycle Time**

Estimation of the cell cycle time ($T_c$) was made from a rate of metaphase accumulation after addition of colchicine.

It has been verified by Puck and Steffen (1963) that the following equation holds between the rate of metaphase accumulation after addition of colchicine and $T_c$,

$$\log (1+m) = \frac{\log 2}{T_c} \cdot t$$

wherein $m$ represents the proportion of cells intercepted in the metaphase, $t$ the time after addition of colchicine. This equation is applicable to the region $T(M) \leq t \leq T_c$, where $T(M)$ is time of mitotic phase.

This relationship, however, is applicable only to such cell populations where all cells are in the process of proliferation, and we employed the following equation wherever the growth fraction might not be 1.0,

$$\log (1+m/f) = \frac{\log 2}{T_c} \cdot t$$

where $f$ represents the growth fraction.

To obtain the value of $T_c$ with this equation, a regression line, $y' = y + b (x - \bar{x})$, was determined by the least square method with log $(1+m/f)$ of the equation as ordinate and with $t$ as abscissa. Thus, the following relationship holds between the regression coefficient $b$ and the cell cycle time $T_c$,

$$T_c = \frac{\log 2}{b}$$

Furthermore, the standard error of estimate $S_e$ of the regression coefficient $b$ was calculated, and 95 per cent confidence interval of $T_c$ was obtained.
In determining $T_c$ by this method, it is desirable to take $t$ as wide as possible within the region $T(M) \leq t \leq T_c$, but, in practice, it is impossible to take the experimental points after the appearance of micronuclei following addition of colchicine. As a result of treatment with colchicine, cells in the culture are intercepted in the stage of metaphase, thereby remaining for several hours in a state of so-called c-mitosis. Subsequently, a micronucleus is formed from a few chromosomes and the cell becomes to possess a number of micronuclei which fuse in time into a restitution nucleus (Leven 1954). But for appearance of such micronuclei possessing cells by gamma-irradiation alone, there will be no problem at all. However, cells possessing micronuclei occur among gamma-irradiated cells, and only c-mitosis must be subjected to the measurement.

Fig. 2 shows the time courses of proportions of c-mitosis cells and micronuclei possessing cells in a non-irradiated cell population after addition of colchicine. As may be seen, cells possessing micronuclei begin to emerge at 6 hours after addition of colchicine. Since the freshly formed micronuclei are generally increased in number and hyperchromatic, they are distinguishable from cells in which micronuclei have formed owing to irradiation. In view of this, the cell cycle time was estimated from the rate of metaphase accumulation up to 7 hours after addition of colchicine.

Fig. 3 illustrates, as an example, the results of application of this method to a non-irradiated cell population and also to a cell population which was exposed to 700 R three days (67-74 hrs) previously. As the regression coefficient $b$ had been determined to be $2.94 \times 10^{-2}$ for the non-irradiated cells and $2.34 \times 10^{-2}$ for the cell population which was exposed to 700R, the median cell cycle times were estimated to be 10.2 and 12.9 hours, respectively. Furthermore, insomuch as the standard errors of estimate $S_e$ of the regression coefficient $b$ were $3.31 \times 10^{-3}$ and $8.50 \times 10^{-3}$, respectively, 95 per cent confidence intervals of the median cell cycle time were inferred to range from 9.8 to 10.7 hours and from 11.9 to 14.1 hours, respectively.

![Fig. 2. Accumulation of metaphase cells and cells possessing micronuclei in a non-irradiated cell population treated with colchicine.](image-url)
Fig. 3. Regression lines of log (1+m/f) against time after addition of colchicine. A, non-irradiated; and B, irradiated with a dose of 700R at 67 hr previously.

The values obtained by this method are considered to be not the mean, but the median of cell cycle time, as is the rule with the labeled mitosis method (Quastler 1963).

RESULTS

1) Dose–survival relationship

Fig. 4 shows the dose-survival relationship on a criterion of macroscopic colony forming ability. As is evident from the curve, the mean lethal dose (Do) for this cell line was noted to be 150 R and the extrapolation number (n) to be 1.5.

The kinetic analyses were performed thereafter with cell populations irradiated with 500 R (survival fraction: \(5.3 \times 10^{-2}\)) and with 700 R (survival fraction: \(1.5 \times 10^{-2}\)).

Fig. 4. Dose–survival relationship determined from macroscopic colony forming ability. Radiosensitivity of this cell line can be expressed as \(D_0=150\) R, \(n=1.5\).

2) Growth curves

Fig. 5 shows time courses of the total cell number after gamma-irradiation. Three separate sets of experiment were carried out with respective dose, and the...
averages of relative cell number are shown in Fig. 5. In view of the doubling time for non-irradiated cells which was estimated by repeated experiments to be approximately 11 hours, the growth curve of non-irradiated cells is rectilinear.

The result of an experiment conducted to determine the mitotic delay is presented in Fig. 6. Colchicine was added to the culture at 2 hours after irradiation and, thereby, reappearance of mitotic figures and pattern of its accumulation were sought. We estimated from the data the duration of mitotic delay to be 5.5 hours in the case of 500 R and to be 8 hours in the case of 700 R.

![Fig. 5. Growth curves of gamma-irradiated cells.](image)

![Fig. 6. Mitotic delay after irradiation. Colchicine was added to culture at 2 hr after irradiation.](image)

Characteristics of the growth pattern shown in Fig. 5 are as follows: The increase in the number of cells is not drastically inhibited for some time after released from mitotic delay. The rates of increase in total cell number were noted to be minimal about three days after irradiation in both cases, the rates increased gradually thereafter to approach the control level.
3) The growth fraction and the dead cell fraction

The growth fractions of the gamma-irradiated populations were determined by the above-mentioned method, and these are summarized in Table 1, in which the dead cell fractions of the gamma-irradiated populations when the growth fractions were determined are also shown. Figs. 7 and 8 were obtained by plotting the values for the growth fraction and the dead cell fraction against time after gamma-irradiation, respectively.

<table>
<thead>
<tr>
<th>Hours after irradiation</th>
<th>Growth fraction</th>
<th>Hours after irradiation</th>
<th>Dead cell fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>0.863</td>
<td>22</td>
<td>0.070</td>
</tr>
<tr>
<td>44</td>
<td>0.614</td>
<td>44</td>
<td>0.154</td>
</tr>
<tr>
<td>71</td>
<td>0.524</td>
<td>70</td>
<td>0.265</td>
</tr>
<tr>
<td>93</td>
<td>0.654</td>
<td>91.5</td>
<td>0.233</td>
</tr>
<tr>
<td>116</td>
<td>0.847</td>
<td>116</td>
<td>0.217</td>
</tr>
<tr>
<td>142</td>
<td>0.171</td>
<td></td>
<td></td>
</tr>
<tr>
<td>165</td>
<td>0.925</td>
<td>166.5</td>
<td>0.103</td>
</tr>
<tr>
<td>190</td>
<td>0.070</td>
<td></td>
<td></td>
</tr>
<tr>
<td>214</td>
<td>0.933</td>
<td>212</td>
<td>0.059</td>
</tr>
<tr>
<td>237.5</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The growth fraction of the irradiated cell population was observed to become minimum on the 3rd day of irradiation. It was inferred, for example, that only about a half of the cells in population had proliferated at this time after received an irradiation of 700 R. Another important feature is that a considerable length of time was required for the growth fraction to return to the control level.

In Fig. 9, the living cell fraction and the growth fraction in the irradiated cell population are compared. This seems to imply a possible presence of cells which are living but non-proliferating, the quiescent cells, in the cell population that has been exposed to gamma rays. Fig. 10 shows time courses of the quiescent cell
Fig. 7. Time course of the growth fraction in the gamma-irradiated cell population. The growth fraction was estimated from labeling indices after time-different addition of $^3$H-thymidine and colchicine.

Fig. 8. The dead cell fraction of the irradiated cell population.

Fig. 9. Comparison of the growth fraction (GF) and the living cell fraction (LF) in cells irradiated with 700 R.
fraction after irradiation as calculated from the data presented in Table 1. As can be seen, an increase of the quiescent cell fraction preceded that of the dead cell fraction (Figs. 8 and 10).

Thus, it is evident that diminution of the growth fraction that takes place in the irradiated cell population is attributable to appearance of quiescent cells as well as dead cells.

4) **Cell cycle time**

The median cell cycle time of proliferating cells at various times after irradiation was estimated by the above-mentioned method. Table 2 summarizes

**Table 2. The median cell cycle time of gamma-irradiated cells**

<table>
<thead>
<tr>
<th>Hours after irradiation</th>
<th>Regression line*</th>
<th>Cell cycle time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression coefficient (b)</td>
<td>Standard error of estimate (SE)</td>
</tr>
<tr>
<td></td>
<td>2.94×10⁻²</td>
<td>3.31×10⁻³</td>
</tr>
<tr>
<td>Non-irradiated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>700 R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19–25</td>
<td>2.61×10⁻²</td>
<td>6.21×10⁻³</td>
</tr>
<tr>
<td>42–47</td>
<td>2.63×10⁻²</td>
<td>7.25×10⁻³</td>
</tr>
<tr>
<td>67–74</td>
<td>2.34×10⁻²</td>
<td>8.50×10⁻³</td>
</tr>
<tr>
<td>91–97</td>
<td>2.33×10⁻²</td>
<td>9.88×10⁻³</td>
</tr>
<tr>
<td>114–119</td>
<td>2.53×10⁻²</td>
<td>5.24×10⁻³</td>
</tr>
<tr>
<td>136–168</td>
<td>2.49×10⁻²</td>
<td>8.67×10⁻³</td>
</tr>
<tr>
<td>213–217.5</td>
<td>2.66×10⁻²</td>
<td>9.58×10⁻³</td>
</tr>
<tr>
<td>500 R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16–22</td>
<td>2.73×10⁻²</td>
<td>4.19×10⁻³</td>
</tr>
<tr>
<td>40–45.5</td>
<td>2.59×10⁻²</td>
<td>5.78×10⁻³</td>
</tr>
<tr>
<td>65–69</td>
<td>2.38×10⁻²</td>
<td>4.04×10⁻³</td>
</tr>
<tr>
<td>87–92.5</td>
<td>2.76×10⁻²</td>
<td>13.3×10⁻³</td>
</tr>
<tr>
<td>113–117.5</td>
<td>3.01×10⁻²</td>
<td>8.48×10⁻³</td>
</tr>
<tr>
<td>136–142</td>
<td>2.88×10⁻²</td>
<td>4.36×10⁻³</td>
</tr>
<tr>
<td>166–166</td>
<td>2.92×10⁻²</td>
<td>3.34×10⁻³</td>
</tr>
</tbody>
</table>

* Regression line of log(1+m/f) against t, where m is the c-mitosis fraction; t is time after addition of colchicine; and f is the growth fraction.
the data thus obtained with respect to the median cell cycle time and its 95 per cent confidence interval, which were determined from the regression coefficient $b$ and its standard error of estimate $Se$ of the regression line. Fig. 11 illustrates time courses of the median cell cycle time and its 95 per cent confidence interval after irradiation.

There is an increase, though not conspicuous, in the median cell cycle time of proliferating cells in the irradiated cell population, as compared with that of proliferating cells in a non-irradiated population. On regression curve analysis, the regression coefficient $b$ for cells irradiated with 700 R was noted to be significantly smaller ($\alpha=0.05$) at any examined time after irradiation than that for cells in non-irradiated culture. Nevertheless, the differences are not saliently great, as shown in Table 2.

![Fig. 11. Median cell cycle time and its 95 per cent confidence interval of the proliferating cells at various times after irradiation.](image)

**DISCUSSION**

A maximal reduction in the rate of increase in total cell number was found to occur characteristically after a lapse of considerable time following gamma-irradiation with doses of 500 or 700 R in FM3A cell population. Similar growth patterns have been observed with cultured cells (Elkind and Whitmore 1967), ascites tumor cells (Sasaki 1969), and solid tumor cells (Thomlinson and Craddock 1967) exposed to the ionizing radiation. The phenomena thus appear to be universal.

It is generally believed that suppression of the rate of increase in total cell number is given rise to by one or more of such possible factors as (1) prolongation of the cell cycle time, (2) decrease in the growth fraction, and (3) increase in cell loss. The diminution of cell growth rate observed in FM3A cell population which had been irradiated with 500 or 700 R may be largely due to decrease in the growth
fraction and only modestly attributable to prolongation of cell cycle time.

The above finding that gamma-irradiation caused an unconspicuous prolongation of the cell cycle time, is consistent with that by Elkind et al. (1963) and by Sasaki (1969), and those by time-lapse cinematography (Thompson and Suit 1967, Hurwitz and Tolmach 1969). It is practicable to determine the median cell cycle time, but impracticable to measure the deviation in this analysis whereby the cell cycle time is estimated from the rate of metaphase accumulation by colchicine treatment. The degree of deviation, therefore, should be determined by other method.

The experimental data obtained suggest that the quiescent cells occur in a fairly high incidence in the irradiated cell population. Quiescent cells are known to be present in solid tumors and in highly dense populations of ascites tumor cells, and these cells retain their ability to return to the cell cycle when put in an environment suitable for proliferation (Lala and Patt 1966). In the present experiments, however, where the culture media were maintained constantly in the best condition, the quiescent cells might be destined to die. The fact that increment of the dead cell fraction was preceded by increase in the quiescent cell fraction seems to provide evidence in support to the above fact. It is an important problem whether the quiescent cells are entirely devoid of the capacity to synthesize DNA or perform a markedly suppressed DNA synthesis. The latter possibility cannot be denied.

Departure from the cell cycle and/or death of cells are a direct cause for deprivation of the so-called macroscopic colony forming ability and can be interpreted as an expression of lethal damage. More precise understandings of the kinetics of departure from cell cycle and of cell death are necessary in order to approach the problem of delayed expression of radiation-induced lethal damage.

Acknowledgment

The author would like to acknowledge the helpful discussion of Prof. M. Sakka. This investigation was supported in part by a Special Grant in Aid for Cancer Research from the Ministry of Education, and in part by a grant for research provided by the Waksman Foundation in Japan.

References

Proliferation Kinetics of Irradiated Cells