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Rapid Communication

Nick Translation Detection *In Situ* of Cellular DNA Strand Break Induced by Radiation

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DNA strand break in HeLa cells induced by radiation was detected using the* in situ *nick translation method. The cells were exposed to radiation of 3, 6, 12, 18, and 24 Gy in Lab-Tek tissue culture chamber/slides and were fixed with ethanol/acetic acid on the slide glass. The break sites in DNA were translated artificially in the presence of* Escherichia coli *DNA polymerase I and [^{3}H]-labeled dTTP. Autoradiographic observation was made of the level of break sites in the DNA. The DNA strand break appeared even with a 3 Gy exposure, increased 8.6 times at 24 Gy compared with the control cells, and this level correlated reciprocally to change in cell viability. This nick translation method provides a rapid* in situ *assay for determining radiation-induced DNA damage of cultured cells, in a semiquantitative manner. (Am J Pathol 1989, 134:7–10)

When determining the lethal effect of radiation, DNA is considered to be the most critical target.[1] Cellular DNA strand break has been observed in radiation-treated cells using the alkaline unwinding method,[1] alkaline elution method,[2] or fluorometric analysis.[3,4] Because these techniques involve the use of a fair number of cells and the destruction of cells for *in vitro* analysis, the morphology of the individual cell can hardly be distinguished. Therefore, detection of DNA strand break on fixed cells using an *in situ* method would appear to be appropriate. The nick translation method is used widely for radioisotope labeling of DNA probes in gene technology[5] and has been used to detect DNA strand breaks in carcinogen-treated cells.[6,7] Iseki and Mori reported that the nick translation method facilitates visualization of DNA strand breaks in bleomycin-exposed HeLa cells[8] and also in murine normal tissues.[9] Kerem et al[10] applied this method to differentiate between active and inactive X chromosomes. Using this method, we found that the DNA break in HeLa cells is triggered by heat (43 C) within 15 minutes.[11] Thus, use of the nick translation method makes a quantitative determination of DNA strand breaks feasible. We report here the *in situ* detection of DNA strand breaks after exposure of HeLa cells to radiation, using this method.

Materials and Methods

Cell Culture

HeLa cells were routinely cultured in monolayer on plastic dishes, using minimal essential medium with *L*-glutamine (292 mg/ml), 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamycin (40 μg/ml). For determining cell viability with the succinate dehydrogenase inhibition (SDI) test,[12] 3×10^5 cells were plated in 60 mm plastic dishes and incubated at 37 C in a humidified 5% CO_2 atmosphere for 2 days. For the nick translation assay, the cells were grown in Lab-Tek tissue culture chamber/slides (Miles Scientific) for 2 days.

X Irradiation

X irradiation was given with a conventional x-ray generator run at 200 kV(p), 20 mA, with 0.3 mm Cu + 1.0 mm Al

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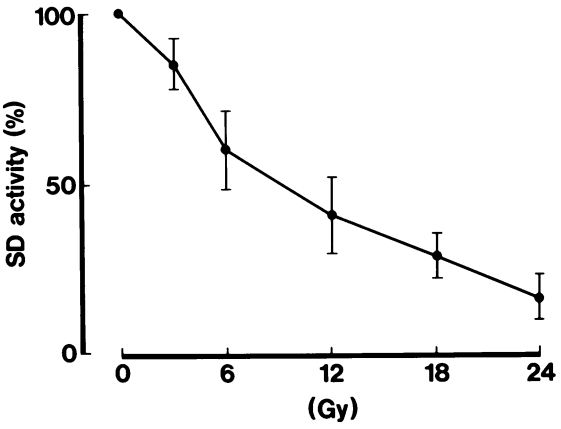


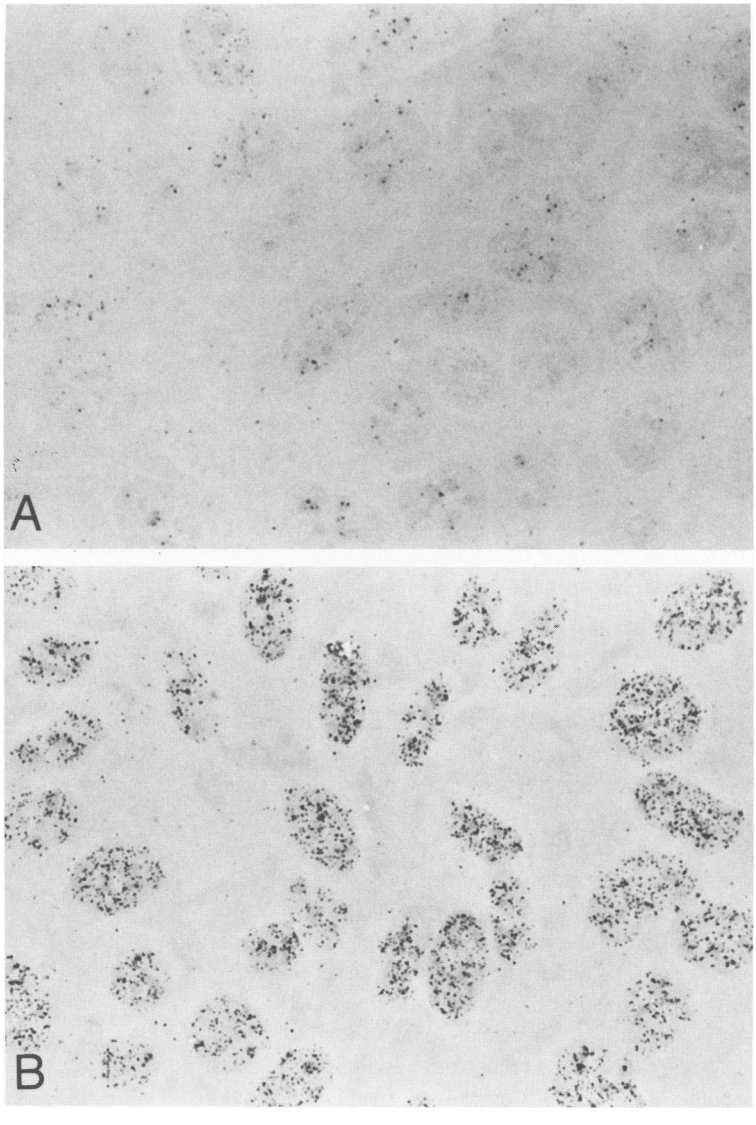
Figure 1. *Change of cell viability in HeLa cells exposed to radiation. Three × 10^5 cells were exposed to radiation at a dose of 3, 6, 12, 18, or 24 Gy and cell viability was determined on day 3 using the SDI test.*

filter at a dose of 0.306 Gy/min at 25 C.[13] After incubation for 2 days, the cultured cells were irradiated at 3, 6, 12, 18, or 24 Gy, and were incubated for 3 days for the SDI test. For the nick translation assay, the plastic chambers were removed from the slides immediately after exposure to radiation and the slides were then rinsed with phosphate-buffered saline (PBS) and fixed for 10 minutes with ethanol/acetic acid (3/1, vol/vol), at room temperature. Each assay was performed in triplicate.

SDI Test

The reaction mixture,[12] in a total volume of 1 ml, consisted of PBS, 10 mM sodium succinate, and 0.04% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H tetrazolium bromide

Figure 2. *Visualization of* in situ *nick translation. After nick translation* in situ *with [^{3}H]-labeled dTTP, HeLa cells were autoradiographed and stained with hematoxylin.* **A:** *Cells without treatment.* **B:** *cells exposed to radiation at 24 Gy. Original magnification ×597.*



(MTT)[14-17] as the hydrogen acceptor. After incubation at 37 C for 1 hour, the cells in the reaction mixture were precipitated by centrifugation, the formed formazan was extracted with 3 ml of acetone containing 0.5% trichloroacetic acid, and absorbance of formazan was measured at 565 nm, using a spectrophotometer (Beckman Co.).

In Situ *Nick Translation and Staining*

After fixation of the cells, the slides were incubated at room temperature for 10 minutes with a 15 μl nick translation mixture containing 50 mM TRIS-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 U/ml of *Escherichia coli* DNA polymerase I (endonuclease-free, Boehringer Mannheim GmbH, West Germany), 30 μM each of dATP, dGTP, dCTP, and dTTP (Sigma Chemical Co., St. Louis, MO), and 1 μM [^{3}H]-labeled dTTP (118 Ci/mmol, Amersham, Japan).[8,9] The reaction was terminated by washing the slides with 50 mM TRIS-HCl, pH 7.5. The slides were then rinsed in ethanol, dried, covered with photographic emulsion (Sakura NR-M2, Konishiroku Photo Ind. Co., Japan), and stored in the dark at 4 C for 3 days. The autoradiographs were developed and the nuclei were stained with hematoxylin.

Densitometer Tracing

The grain density in each nucleus was analyzed microscopically using an image processor (EXCEL, Nippon Avionics Co., Japan) and 100 nuclei were used for counting, in each slide.[11] The ratio of densities between the radiation-treated and control cells was determined.

Results

When HeLa cells were exposed to radiation of various doses, the surviving fraction decreased, as assessed by the SDI test, in a dose-dependent manner, as shown in Figure 1. The SD activity decreased to 84.9% for 3 Gy, 59.9% for 6 Gy, 40.8% for 12 Gy, 28.3% for 18 Gy, and 16.4% for 24 Gy compared with control cells on day 3. *In situ* nick translation led to visible evidence of DNA strand breaks after radiation. When the cells incubated at 37 C without radiation were nick translated in the presence of DNA polymerase I, the nuclei had only a few grains: 9.6 ± 2.5 grains in a nucleus, in an average of 100 nuclei (Figure 2A). However, when the cells exposed to radiation at 24 Gy were nick translated, the nuclei had a number of grains (Figure 2B). The increase in grains was 1.8-fold at 3 Gy, 2.8-fold at 6 Gy, 3.9-fold at 12 Gy, 7.1-fold at 18 Gy, and 8.6-fold at 24 Gy compared with findings for control cells, as determined by an image processor (Figure 3).

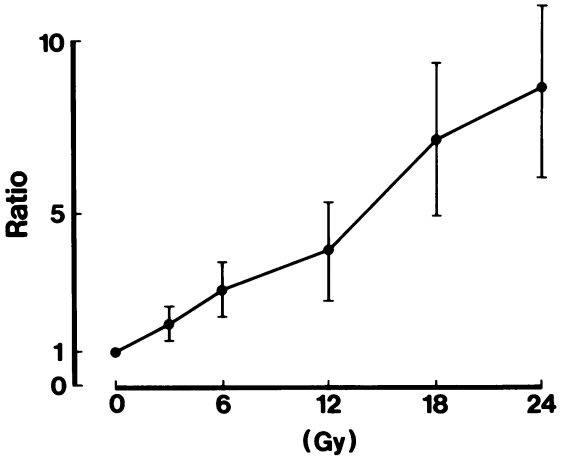


Figure 3. *Increase of the DNA strand break in HeLa cells exposed to radiation. Ratio of grain densities between the radiation-treated cells and the control cells was determined using an image processor. One hundred cells were used to count grain density. Values are mean ± standard deviation.*

Discussion

Elucidation of the molecular mechanism of radiation-induced cell killing is important because radiotherapy for clinical cancer is now in wide use. The biochemical effects of radiation on malignant cells were noted and damage to the DNA was found to closely relate to radiation-induced cell killing.[1-4] Early detection of changes in cellular DNA induced by radiation should aid in designing radiotherapy for an individual patient. With the alkaline unwinding method,[1] the DNA break is seen as a change in binding to hydroxyapatite, in sensitivity to an enzyme, or in binding of a fluorescent dye. In the alkaline elution method,[2] the DNA break is detected as an increased rate of passage of DNA through a filter. The fluorometric analysis[3,4] is particularly well suited to the study of nondividing cells, such as lymphocytes, the DNA of which cannot readily be radiolabeled. These methods need a fair number of cells, however, and the morphology of the cell is necessarily destroyed.

Using the *in situ* nick translation method,[8,9] we found that the DNA strand break appeared at 3 Gy in HeLa cells and the number of breaks could then be determined semiquantitatively. Thus, this method is applicable for detecting DNA damage induced by radiation. Because the decrease of cell viability after exposure to radiation has been determined using the radiosensitivity test (MTT assay[17]) the reciprocal correlation between the level of DNA strand breaks immediately after radiation and the ensuing decreased cell viability suggests that the DNA strand breaks

relate to radiation-induced cell killing. Because the level of radiosensitivity differs with the cell species,[17] the *in situ* nick translation method is meaningful for predicting the effect of radiotherapy.

The *in situ* nick translation method is technically rapid and cost-effective. Only a small number of cells are needed. Cell morphology remains intact. It can be used for a wide variety of nondividing or slowly dividing cell types, and is predictable for assessing radiation susceptibility, hyperthermia susceptibility,[11] and chemical agent susceptibility of mammalian cells.

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