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Apoptosis and necrosis of HeLa cells in response to low-energy ion radiation

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Abstract The aim of this study was to investigate the damage of low-energy ions to HeLa cells and to particularly examine the relationship between apoptotic and necrotic effects and the low-energy ion radiation. In this study, HeLa cells were irradiated by low-energy ions (30keV N^+) at different doses. The level of apoptosis and necrosis was evaluated using flow cytometry. Since vacuum is required for experimental low-energy ion generation and irradiation, the cells must be placed in vacuum. Mineral oil was used to prevent dehydration of cells. The results show that the apoptotic rate reached 7.09% when the ion implantation dose was 1×10^{15} ions/cm^2; and when the cells were exposed to and implanted at 2×10^{15} ions/cm^2 dose, the apoptotic rate was higher than that at 1×10^{15} ions/cm^2, and the necrotic rate was 15.63%. In addition, the survival fraction gradually decreased with the increase in implantation dose. Some relationships have been found between the radiation-induced apoptosis and the incubated time after irradiation.

Key words Low-energy ion, Apoptosis, Necrosis, Flow cytometry

CLC number Q345$^+$.2, Q274

1 Introduction

The biological effects of the different kinds of radiation such as photons, α particles, electrons, and protons have been widely studied over 100 years. However, the interaction between the low-energy ions (with energy lower than 100 keV) and organisms was uncertain. Based on the concept that ions will penetrate into a dense matter, the depth range of the incident ions with such low energies is rather limited, and these may have little or no effect on organisms [1]. However, the depth of the ions penetrating into the plant cell-wall has been measured, and the range of ions in the cell wall is considerably greater than those for a solid [2]. Qualitative studies on ion-beam etching of rice cell walls have shown heavy sputtering of ions when the cells were bombarded with 30 keV N^+ or Ar^+ ions at a fluence of 1.5×10^{15} ions/cm^2 [3,4]. It has been proposed that significant dilution of the cell wall by ion-beam sputtering could enhance the cell-wall permeability.

In fact, more than 90% of the electriferous particles with low energy are from solar wind and interstellar molecules. Low-energy ions could be generated on the earth's surface through many processes such as thunderbolt, static, by radioactive elements transmutation, and by volcanic eruption. Various studies have proved that low-energy ions can induce complicated effects in plants, microbes, naked DNA, and organic molecules. Recent studies have revealed the possibility that low-energy ions play an important role in the origin and evolution of life, and therefore, low-energy ion radiation should not be ignored [1].

Radiation-induced apoptosis or necrosis has been extensively studied. In the present study, the apoptotic and necrotic effects of low-energy ion irradiation were

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measured on mammalian cells, which can be used for evaluating the effect of low-energy particles on the environment.

Since vacuum is required for low-energy ion generation and irradiation in experiments, mineral oil was selected to protect the cells from dehydration. This is because the tolerance of mammalian cells to vacuum (10^{-2} Pa pressure) is enhanced efficiently after the cells were pretreated with mineral oil, which made it possible to study the biological effects after the ion beam implants in the mammalian cells.

2 Materials and methods

2.1 Cell culture

HeLa cells were cultured in the RPMI 1640 medium (GIBCO BRL Cat. No 31800-022) with 10% fetal bovine serum and were incubated in air with 5% CO_2 at 37°C. The cells were subcultured at a density of 10^5/mL in a total volume of 10 mL of the medium. The cells before vacuum exposure and ion bombardment were placed in the ϕ 60 mm tissue-culture dishes and were incubated overnight.

2.2 Implantation condition

Low-energy ions were generated and radiated by The Titan (Russia), which consisted of ion source, pumping system, vacuum chamber, and control systems. This was developed specifically for the ion bombardment of biological materials. The bombardment parameters could be regulated in advance, and the ion bombardment was carried out sequentially. In this study, the vacuum pressure was 5×10^{-2} Pa, and the ion (N^+) energy was 30 keV.

2.3 Sample treatment

The HeLa cells were marinated in 100% mineral oil (Sigma Lot 21k0030) for 10 min before treatment. The studies indicated that the 100%-mineral oil had no obvious toxicity to the HeLa cells when the cells were marinated for 2–10 min [5]. Oil was then poured completely from the dishes when the cell dishes were placed in the sample chamber.

2.4 Fluorescent staining

After vacuum or implantation, the HeLa cells were digested, and the cell density was regulated at 5×10^5 ~1×10^6 /mL. The cells were then resuspended in 200 μL of binding buffer, and the cell suspension was mixed with 10 μL of Annexin V-FITC and 5 μL of PI (Biosea Biotechnology, Beijing, CX1001), which reacted at 4°C in dark for 30 min. Finally, 300 μL of binding buffer was added to the cell suspension, and the samples were analyzed using flow cytometry in 1 hour.

2.5 Flow cytometry

Flow cytometry analysis was performed by an FACSVtage SE (BD, US) equipped with argon ion laser, which emitted 488nm(300mW) line for exciting Annexin V-FITC (green fluorescence) and PI (red fluorescence). The staining assay was easy to perform and distinguished intact cells (FITC-/PI-), apoptotic cells (FITC+/PI-), and necrotic cells (FITC+/PI+) [5]. The data were analyzed using the Becton Dickinson CellQuest program.

3 Results

3.1 Induction of apoptosis and necrosis by low-energy ion irradiation

Apoptosis was measured with the percentage of Annexin V-positive and PI-negative cells, and necrosis was measured with the percentage of both Annexin V-positive and PI-positive cells using a flow cytometer (Fig.1). It showed that the apoptotic level reached 7.09% when the implantation dose was 1×10^{15} ions/cm^2, that is, the implantation time of the ions was 64 s in vacuum, whereas the apoptotic level was higher at an implantation dose of 2×10^{15} ions/cm^2. However, the apoptotic percentage was 0.67% after 2 min in the simple vacuum without ions, and the value was only 0.83% after 5 min in the simple vacuum without ions.

Fig. 1 indicates that both vacuum and implantation can induce necrosis but to different levels. When cells were implanted at 2×10^{15} ions/cm^2, and the ions implantation time was 128 s in vacuum, the necrotic percentage was 15.63%, which was considerably more than that in simple vacuum for 2 min without ions, even for 5 min. It can be seen that the low-energy ion radiation can induce not only apoptosis but also necrosis.

In addition, the apoptotic level in the simple vacuum environment without ions was similar to that in

control, whereas the necrotic level in simple vacuum was considerably higher than that in control. Therefore, the main effect of vacuum was to induce necrosis, while the apoptotic effect was induced almost completely by the low-energy ions and not by vacuum.

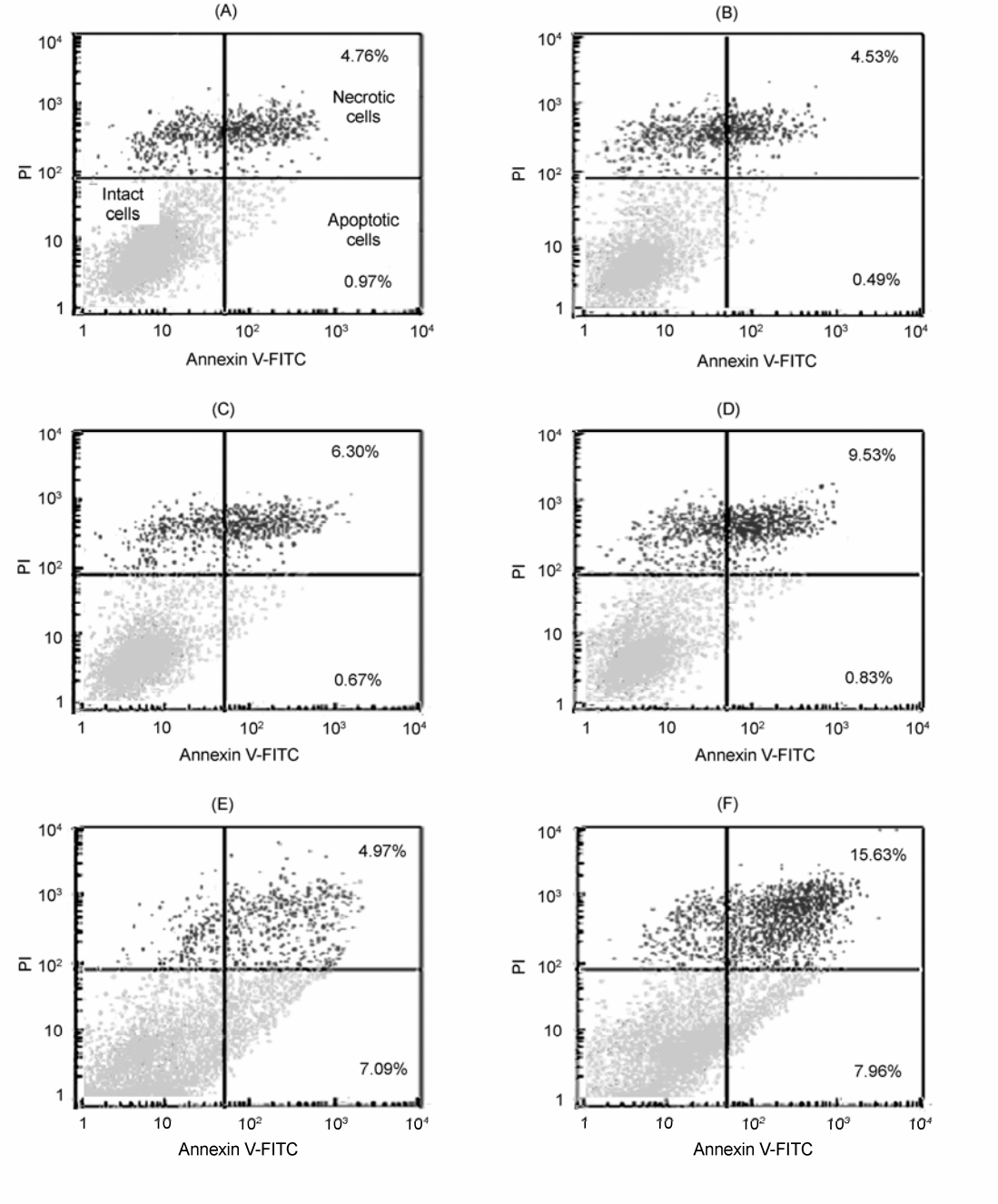

Fig.1 The apoptotic or necrotic levels.

(A) Control. (B) Cells marinated in mineral oil for 10 min . (C) Cells in simple vacuum without ions for 2 min after pretreatment. (D) Cells in simple vacuum without ions for 5 min after pretreatment. (E) Cells implanted at 1×10^{15} ions/cm^2 after pretreatment. (F) Cells implanted at 2×10^{15} ions/cm^2 after pretreatment.

3.2 Survival–dose graph

The survial–dose graph (Fig.2) was plotted according to the data measured using flow cytometry. In this graph, the number of dead cells (of both apoptosis and necrosis) increased with the dose. When the implantation dose was 3×10^{16} ions/cm^2, the survival fraction was only 43.84%, suggesting that the damage level was enhanced gradually with the increase in dose.

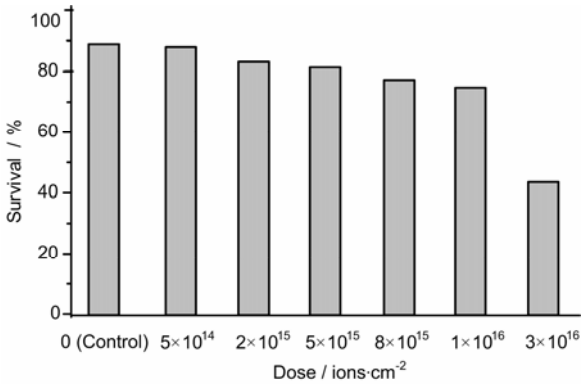

Fig. 2 Survival–dose graph of the HeLa cells implanted by N^+.

3.3 Apoptotic levels for different post irradiation times

An obvious reduction in apoptosis was observed in the course of incubation for 72 h post irradiation (Fig.3). When the cells were incubated for 48 h after exposure, the apoptotic level was found to be either higher or lower than that at 0 h.

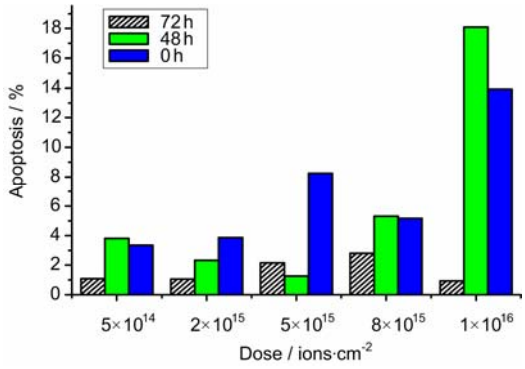

Fig.3 Apoptotic levels of HeLa cells at different implantation doses for different post irradiation incubation times.

4 Discussion

Apoptosis and necrosis were measured using flow cytometer. However, the apoptotic cells could eventually lose their ability to exclude PI, which could lead to an underestimation of the actual number of apoptotic cells. Thus, morphological analysis was needed to identify the necrotic cells from the apoptotic cells [6].

Apoptosis plays a vital role in the development of organisms and in the renewal of tissues. Recent data also indicate that necrosis appears to be a specific form of the execution phase of programmed cell-death. Disturbance of a fine balance between necrosis and apoptosis may be a key element in certain diseases [7].

Why were the apoptotic cells after implantation considerably more than that in simple vacuum environment (Fig.1)? Why were the necrotic cells in simple vacuum environment considerably more than the apoptotic cells in the same environment (Fig.1)? This variability may be partly because of the relative contribution of the different mechanisms of cell killing (apoptosis or necrosis) at different rates.

With regard to the first question, it is possible that the implanted ions triggered some mechanism of apoptosis. The target for apoptosis may be the plasma membrane, nuclear DNA, or both [8]. However, according to the power theory, the depth range of 20 keV ions in water is less than 1 μm. A mammalian cell could be seen as a body of water, and the average thickness of a spreading HeLa cell is about 4 μm. Therefore, it is inferred that the implanted ions may not pass through the cells, or may not even reach the nucleus [1]. The radiation ions appeared to directly act on the plasma membrane to induce hydrolysis of sphingomyelin to ceramide, which caused apoptosis [8]. In addition, the implanted ions possibly reacted with some ingredients of cytoplasm and created or damaged the signal molecule or induced the signal molecule to respond in the membrane or cytoplasm to cause apoptosis.

With regard to the second question, necrosis is usually induced by nosogenesis in the environment. In this study, cells were damaged immediately during vacuum exposure, and the damage was very severe. The injury was characterized by alteration of the membrane permeability, loss of firm adhesion to the dish, and increased fragility [9]. Therefore, in such severe environment, most of the cells selected necrosis, cells were more attracted to PI and were stained with both Annexin V-FITC and PI. Thus, a large number of necrotic cells were detected in vacuum.

In this study, some relationships were found between the radiation-induced apoptosis and the incubated time post-irradiation (Fig.3). At 48 h after irradiation, the cells could repair the irradiation damage using their own mechanisms when irradiated by a nonfatal dose; however, the fatal damage could not be repaired, which then further led to apoptosis, and thus the apoptotic level was higher or lower than that at 0 h.

At 72 h after irradiation, the cells passed the repairing period and began to divide. High concentrations of serum contained several growth factors and cytokines in the cell culture medium, which may favor cell division instead of apoptosis [10]. The dead cells after irradiation could excrete cytokines that excited the remaining cells to divide. On the other hand, since some cells were dead, the contact inhibition among the cells disappeared, which enabled them to divide quickly [11]. Thus, the apoptotic percentage did not increase but decreased.

In summary, the low-energy ions can damage human cells, and because these causes apoptosis and necrosis, it is possible that the low-energy ions play an important role in the development of organisms and in the renewal of tissues. Therefore, low-energy ion radiation should not be ignored.

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