The Influence of Radiation Quality on Radiation-induced Hemolysis and Hemoglobin Oxidation of Human Erythrocytes

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INTRODUCTION

The influence of radiation quality on cytological effects is well known. In many instances α-particles have been found to be more effective than γ-rays. This is well documented for the loss of colony-forming ability, mutations, chromosomal aberrations, and neoplastic transformations, where the relative biological effectiveness (RBE) is found to be in the range from 3 to about 10.1,2) There are exceptions in this regard, e.g., with radiosensitive mutants3) or with rat tracheal epithelial cells.4,5) However, for many effects on mammalian cells there is a general trend toward increasing RBE with increasing LET.6)

In this work, we concentrated on investigating the influence of high and low LET radiation on human peripheral erythrocytes. Mature human erythrocytes have no nucleus or any other cellular organelles. A critical target whose damage eventually leads to hemoglobin release, i.e., hemolysis, is the plasma membrane, and the intracellular target of erythrocytes is hemoglobin. In this paper, we studied the effectiveness of α-particles and γ-rays for the induction of hemolysis, oxidation, and denaturation of hemoglobin.

MATERIALS AND METHODS

Materials

Human erythrocytes anticoagulated with acid-citrate-dextrose were obtained from the peripheral blood of healthy adult donors. Erythrocytes were separated from blood plasma and leukocytes by centrifugation and washed four times with physiological saline solution. The buffy coat was aspirated each time. The last centrifugation was carried out for 12 min at 280 g, and the cells were then diluted 1:250 with saline. Two ml of suspension prepared in this manner was pipetted into dishes and left for 2 h in a cold room at 4 °C. The dishes were shaken lightly to remove nonadhering cells, and two ml of physiological saline was then poured into them. Erythrocyte preparations made in this manner formed a monolayer of tightly packed blood cells adhering well to the bottom of the dishes.

In regard to irradiation with α-particles, the bottom of the dishes was composed of 2 µm-thick Mylar foil to which the erythrocytes were attached.7) The diameter of the dishes was 4.5 cm. In regard to γ-radiation, erythrocyte monolayers were formed on plastic Petri dishes, 5 cm in diameter.

Irradiation

α-particles: Dishes of erythrocyte monolayers were placed 6 mm above the α-source, which was 241Am-foil with a fluence of 2 × 10⁶ cm⁻² s⁻¹. The maximum energy of α-particles was 3.5 MeV, the average dose-rate 1.92 kGy/h, and the track-average LET was estimated to be 190 keV/µm at the surface of the cells. The erythrocytes were irradiated at room temperature with doses ranging from 0.9 to 6.8 kGy; and the exposure times ranged from 0.5 h to about 3.5 h.

The α-source used was not collimated so that there was an inconsistent distribution of energy levels and thus also LET
values. This condition was insufficient; it was not part of the main objective of our work, to investigate the effect of radiation quality on membrane damage, since the lowest LET found in the spectrum was about 100 keV/μm. It should also be noted that collimated α-beams have only recently become available and that most of the work to which we refer were performed with α-sources similar to ours.

γ-rays: Irradiation with γ-rays was performed on Petri dishes using a 60Co-source with a dose-rate of 5.8 kGy/h, determined by a modified Fricke dosimetry as described previously.30 Erythrocytes were irradiated at room temperature with doses ranging from 0.9 to 5.8 kGy. The maximal time exposure was equal to 1.0 h.

Determination of erythrocytes hemolysis

Percentages of hemolysis were determined by the measurement of hemoglobin (Hb) released from the cells, relative to the total cellular Hb content. After irradiation, the supernatants were carefully aspirated from the erythrocyte monolayer and then centrifuged. The remaining erythrocytes were then hemolyzed by adding of 2 ml of distilled water to the surface of the dish. Absorption spectra were recorded in the range of 470–700 nm. The hemolysis, Fe(III) content and the level of hemoglobin denaturation in erythrocytes were determined.

The percentage of hemolysis was calculated with the following equation:

\[ H(\%) = \frac{A_1}{A_1 + A_2} \times 100\% \]

where: \( A_1 \) absorbance of the Hb in the supernatant; \( A_2 \) absorbance of the Hb solution after total hemolysis.

The absorbance of these solutions was measured at 522 nm (the isobestic point for HbO2 and MetHb). All incubations were done in the air at room temperature.

Calculations of Fe(III) content and the degree of hemoglobin denaturation

The percentage of Fe(III) content was calculated on the basis of absorbance values at 630 nm30 with the following

\[ Fe(\%) = \frac{(A_p - A_0)}{(A_p - A_0)} \times 100\% \]

where: \( A_p \) absorbance of the examined solution; \( A_0 \) absorbance of the solution that contains 100% HbO2 [0% Fe(III)]; \( A_0 \) absorbance of the solution totally converted into MetHb [100% Fe(III)].

The level of hemoglobin denaturation was assessed by using the “γ” parameter (\( γ = A_{360}/A_{530} \)) determined for totally oxidized solutions with \( K_{3}[Fe(CN)_6]_n \) and indicative of the extent of its conversion into hemichromes.10 All results are expressed as mean ± SD values of 5 to 7 independent experiments.

Fig. 1. Dose-response for hemolysis of human erythrocytes induced by γ-radiation and α-particles measured about 1 min after exposure. Each point represents the mean of 5–7 separate experiments ± SD.

RESULTS

Erythrocytes lose their viability by membrane damage and release hemoglobin into the medium, i.e., hemolysis, which can also be considered as a special case of “interphase cell death.”11 Figure 1 shows the dose response for hemolysis (the relative fraction of hemoglobin in intact cells 1 min after irradiation). The shape is very similar to that found in conventional colony-forming assays (cfa). The doses, however, are much larger than those needed for a comparable reduction of cfa in mammalian cells. More important, α-particles are clearly “less” effective than γ-rays, at least in the higher dose range over 4 kGy, which is quite contrary to the situation found in the destruction of reproductive capacity (cell death). The parameters of the survival curves are:

α-particles: \( D_{57} = 3.90 \) kGy; \( D_{10} = 1.44 \) kGy; γ-rays: \( D_{4} = 3.87 \) kGy; \( D_{5} = 0.58 \) kGy.

Although the RBE is close to 1 in the low dose range as judged from the \( D_{57} \)-values, it is only 0.4 if the \( D_{10} \)-values are compared. The above values have only descriptive meanings; they do not imply a functional relationship.

At doses lower than \( D_{57} \), radiation-induced hemolysis is not an immediate process, but it sustains upon further incubation.12 The time course analysis may provide further evidence for underlying mechanisms. Figures 2 and 3 exhibit hemolysis as a function of incubation time after irradiation with α-particles and γ-rays, respectively. In Fig. 2, the extent of hemolysis increases proportionally to the incubation time, up to the plateau after irradiation of 3–7 h, depending on the dose. With γ-rays (Fig. 3), however, the curves are sigmoidal and the saturation is already apparent after irradiation of 40–90 min. The curve shapes and the kinetic behaviors both indicate a large difference between the two radiation sources.

The third parameter related to erythrocyte damage, but not
Effect of Radiation on Erythrocytes

Fig. 2. Percent hemolysis of human erythrocytes as a function of incubation time after irradiation by $\alpha$-particles.

Fig. 3. Percent hemolysis of human erythrocytes as a function of incubation time after irradiation by $\gamma$-rays.

Fig. 4. Absorption spectra of hemoglobin (Hb) obtained from $\alpha$-irradiated erythrocytes (dose of 5.76 kGy): 1-Hb released from erythrocytes during radiation, 2-Hb obtained from irradiated and nonhemolyzed cells.

Fig. 5. Absorption spectra of hemoglobin from nonirradiated and $\gamma$-irradiated erythrocytes (dose of 3.87 kGy): c-Hb obtained from nonirradiated erythrocytes, 1-Hb released from erythrocytes during radiation, 2-Hb from irradiated and nonhemolyzed cells.

Table 1. Oxidation and denaturation extent of hemoglobin released from erythrocytes during $\gamma$-irradiation and obtained from residual erythrocytes.

<table>
<thead>
<tr>
<th>Dose (kGy)</th>
<th>Hb released from erythrocytes during radiation</th>
<th>Hb from nonhemolyzed erythrocytes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Fe(III) (%)</td>
<td>$t^*$</td>
</tr>
<tr>
<td>0</td>
<td>_</td>
<td>–</td>
</tr>
<tr>
<td>1.93</td>
<td>_</td>
<td>–</td>
</tr>
<tr>
<td>2.90</td>
<td>96.9 ± 5.4</td>
<td>1.52 ± 0.14</td>
</tr>
<tr>
<td>3.87</td>
<td>95.3 ± 4.4</td>
<td>1.56 ± 0.11</td>
</tr>
<tr>
<td>4.83</td>
<td>91.8 ± 3.5</td>
<td>1.51 ± 0.06</td>
</tr>
<tr>
<td>5.80</td>
<td>94.5</td>
<td>1.21</td>
</tr>
</tbody>
</table>

Value represents the mean ± SD from 5–7 independent experiments. $t^* = A(500 \text{ nm})/A(563 \text{ nm})$ after the total oxidation of Fe ($t = 1.1$ for complete denaturation of Hb). _No hemolysis. ^Complete hemolysis.

to membrane integrity, is the extent of hemoglobin oxidation and denaturation. It can easily be assessed by measuring the absorption spectra. The absorption spectra of hemoglobin shown in Fig. 4 have only two bands of absorption: α (576 nm) and β (540 nm), characteristic of HbO2, which indicate that in the case of α-particles no oxidation or hemoglobin denaturation occurs. However, absorption spectra in Fig. 5 apart from bands characteristic of HbO2 have additional bands at 505 nm and 630 nm, indicating the presence of an oxidized form of hemoglobin (MetHb). Thus the processes of oxidation and denaturation of hemoglobin are significant in regard to γ-radiation. Table 1 shows the level of oxidation of iron and parameters characterizing the extent of hemoglobin denaturation after γ-irradiation. The Fe(III) content and the extent of Hb-denaturation were very high in the fraction of released Hb. The hemoglobin inside the erythrocytes was oxidized at a lower degree, and about 90% was sustained in the native form up to 3.87 kGy. The same parameters determined for preparations irradiated by α-particles showed no changes compared to nonirradiated samples.

**DISCUSSION**

Assuming that a human erythrocyte is a suitable model to assess membrane damage, it may then be concluded from the present results that the dependence on radiation quality is different from the one defined for the most DNA damage. The dose response curves shown in Fig. 1 indicate that under the influence of γ-radiation, hemolysis took place about 2.5 times faster than under the influence of α-particles. The time-dependent curves of hemolysis after irradiation, as presented in Figs. 2 and 3, indicate a different course of erythrocyte hemolysis induced by α-particles and γ-rays. The postradiation curves for the hemolysis of erythrocytes irradiated with γ-rays have a sigmoidal shape (Fig. 3). In this instance, hemolysis begins after some time and is dependent on the radiation dose (the higher the dose, the sooner it begins). For similar doses of α-irradiation shown in Fig. 2, the curves show a slight level of hemolysis at the beginning of measurements (the intercepts with ordinate axis). The beginning level of hemolysis must be attributed to the relatively long exposure times during which it already occurs. They were 60, 90, and 120 min for doses of 1.92, 2.88, and 3.84 kGy, respectively. With γ-rays, the irradiation time was 30 min for 2.9 kGy. The intercept with the ordinate axis shown in Fig. 2 may be compared to the extent of hemolysis in Fig. 3 at corresponding total incubation times to estimate the effectiveness of the two radiation qualities. Eight percent hemolysis is obtained with 1.92 kGy α-exposure immediately after irradiation (exposure time 60 min), it should be compared to the 40-min point in Fig. 3 at the same dose (taking into consideration a 20-min exposure time with γ-rays). Here a value of about 20% is found, which is more than twice higher. A similar estimation can be made for 2.9 kGy yielding 12% for α-particles and 100% for γ-rays, respectively. This estimation also demonstrates that at shorter times α-particles are clearly less effective than γ-rays.

The results obtained in this study indicate the various mechanisms damaging erythrocyte plasma membrane by γ-rays and α-particles. It can be inferred from earlier works that hemolysis induced by γ- and X-rays under air is preceded by intensive processes of proteins and lipids oxidation. These processes are mainly induced by hydroxyl radicals. The yield is considerably smaller with α-particles (G = 0.5), as compared to γ-rays (G = 2.7). It is therefore not surprising that α-particles are found to be less efficient for the membrane damage of erythrocytes. It seems that the indirect effect of water radicals plays only a minor role with α-particles, but it is very substantial with γ-rays. This view was shown by different kinetics of dose-dependent and time-dependent hemolysis after irradiation (Figs. 2 and 3) and the lack of hemoglobin oxidation with α-particles (Fig. 4) as opposed to γ-rays, which showed substantial oxidation (Fig. 5).

Molecular products of water radiolysis, e.g., H2O2, seem to play only a minor role. Since they are formed with higher yields by α-particles (G = 1.1 for α-particles and G = 0.7 for γ-rays) but obviously they are not sufficient to cause significant damage, H2O2 is slightly effective in inducing lipid peroxidation in erythrocyte ghosts and is completely inactive in intact cells because of the presence of endogenous catalase (Puchala, unpublished). It is known that O2 also plays a small role in cell damage; thus the effects observed may be essentially attributed to the smaller yield of OH radicals. The two radiation sources used were not exactly applied at the same dose rate, that of α-particles being about 3 times lower than that of γ-rays. The dose rate plays an important role in radiation-induced membrane damage.20,21 It has been found in our experiments that 10-times–lower dose rate of γ-rays increases the effect to a great degree (data is not show). This means, however, that the RBE of α-particles would be even smaller than described above.

Schiöth et al reported on similar results in human erythrocytes by using accelerated heavy ions that showed RBE values greater than one. However, it is unnecessary to point out that their experiments did not concern α-particles (they didn’t use a helium ion beam) and that they were carried out under different conditions from ours. In our study, monolayers of erythrocytes were irradiated and the hemolysis was measured at a temperature of 22°C, but in the mentioned work the cell suspensions were irradiated and the hemolysis was carried out at a temperature of 0°C; therefore the two sets of experiments cannot be compared.

Current experiments on the inactivation of mammalian cells by single α-particles indicate that hitting the cytoplasm has a very small influence on survival, though the probability of survival decreases as a function of the number of nuclear traversals. Furthermore, some data indicates that the irradiation of liposomes or low-density lipoprotein with high-LET...
particles has induced significantly lower yields of lipid peroxidation products than γ-rays.24,25 Our results can be supported by these previous studies.

In summary, it may be stated that under the conditions used in the experiments described in this paper, the action of α-particles on human erythrocytes in vitro is quantitatively and qualitatively different from that of γ-rays. For all effects measured, γ-rays are "more" effective than α-particles if compared at the same dose level.

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REFERENCES


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