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Effect of Radiation on Red Cell Membrane and Intracellular Oxidative Defense Systems

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Ionizing radiation is currently used for prevention of transfusion associated graft versus host disease (TAGVHD). As radiation damage is associated with the production of activated oxygen species, the aim of this study was to observe the immediate effect of ionizing radiation on red cell membrane and intracellular oxidative defense systems. Neonatal and iron deficiency (IDA) cells, known for their increased sensitivity to oxidative stress, were chosen and compared with normal cells. Irradiation was performed in doses of 1500 cGy, 3000 cGy and 5000 cGy. GSH and methemoglobin levels and the activity of different antioxidant enzymes, measured under optimal in vitro conditions, were preserved in all cells after irradiation. Only radiation at the highest dose of 5000 cGy, caused significant potassium leakage in neonatal cells and insignificant increase in IDA cells. Thus, cells with increased sensitivity to oxidative stress are more susceptible to damage by ionizing radiation than normal cells.

Key words: Radiation, erythrocytes, antioxidant enzymes, potassium

INTRODUCTION

Transfusion associated graft vs. host disease (TAGVHD) is a rare but usually fatal complication following blood transfusion.[1] Ionizing radiation is the most effective means for prophylaxis of TAGVHD.[1] The initial stage of radiation damage in mammalian cells is radiolysis of water. These reactions which involve mainly activated oxygen products, are propagators of damage in irradiated cells.[2,3,4] Radiation may induce severe damage to the cell nuclei, membranes and enzymes.[5] The optimal radiation dose, adequate for TAGVHD prevention, has not yet been established.[6,7] Being devoid of nucleus, the red blood cell (RBC) is a suitable model for the examination of immediate radiation effect on enzymes and membrane: The RBC is well equipped for resisting oxidative stress throughout its normal metabolism.[8–10] Oxidative damage is caused intracellularly when the protective mechanisms are damaged or overwhelmed.[8,11] The aim of this study was to investigate the immediate effect of ionizing radiation on membrane and enzymatic components of the cells.

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MATERIALS AND METHODS

Materials

All chemicals and enzymes were purchased from Sigma, Israel.

Collection of blood samples and radiation procedure

52 samples from packed units, stored 3–10 days, were used as controls. 15 samples of fresh blood were taken from placental vessels of healthy mature newborn babies and 5 samples from IDA patients, according to accepted clinical criteria. 10 ml of each fresh blood were collected into heparinized tubes and transfered into sterile plastic bags supplemented with 1.5 ml of CPDA-1, obtained from Travenol, Ashdod, Israel. 30 ml from packed units were divided equally into 3 groups, transfered into sterile plastic bags and irradiated with doses of 1500 cGy, (570 mev), 3000 cGy (1140 mev), or 5000 cGy (1900 mev). All fresh blood samples were irradiated with 5000 cGy. Irradiation rate was constant and the radiation dose varied by the time of exposure. The irradiation procedure was performed in the Radiation Dept. of Soroka Medical Center with an Accelerator unit. All tests were performed 24 hours after exposure to radiation.

Methods

Hematocrit and hemoglobin values were determined according to accepted laboratory methods.[12]

Methemoglobin (MET) production: cells were mixed with hypotonic phosphate buffer (pH = 6.6), Triton X-100 was added to obtain a clear hemolysate. Absorbance was measured at 630 nm and calculated as percent of total hemoglobin.[13]

Reduced glutathione concentration (GSH): GSH levels were determined by the reduction of DTNB (5'5' Dithionitrobenzoic acid) and the production of a yellow anion. Absorbance was measured at 412 nm. Results are expressed as μmol/gr Hb.[14]

Plasma potassium levels: samples were centrifuged for 3 minutes at 3000 RPM and plasma potassium was determined by flame photometer with appropriate standards.

Enzymatic activity: enzymatic activity of the following enzymes was tested under optimal in vitro conditions. Therefore, these results represent the maximal potential activity of the enzymes examined under in vitro conditions. Superoxide dismutase (SOD) – inhibits the oxidation of pyrogallol, dependent on availability of superoxide anions. The change in absorption per minute is recorded at 420 nm and calculated in relation to a control sample without SOD. Enzymatic activity is expressed in IU/gr Hb.[14]

Catalase (CAT) decomposes hydrogen peroxide. The rate of this reaction is determined by change of absorbance at 230 nm. Results are expressed in IU/gr Hb.[14]

Glutathione Reductase (GR) – reduces oxidized glutathione in a reaction utilizing NADPH. The decrease in NADPH is followed at 340 nm. Results are expressed at IU/gr Hb.[14]

Glutathione peroxidase (GP) – decomposes hydrogen peroxide in a reaction utilizing GSH, which is replenished in the cell by GR. An indirect means for GP activity is to measure the decrease in NADPH at 340 nm. Results are expressed in IU/gr Hb.

Acetylcholine esterase activity (ACE) is an indicator of membrane intactness. The reaction of thiocholine, the product of acetylthiocholine breakdown, with DTNB is measured at 412 nm. Activity is expressed as IU/ml cells.[15] All spectrophotometric methods were performed in a double beam spectrophotometer, spectronic 2000 (Bausch & Lomb, New York). Electron microscopy – Cells were fixed with glutaraldehyde 1% in PBS 0.1 M pH 7.4. The cells were incubated for 1 hour at room temperature, centrifuged, washed and suspended in acetone and observed in a scanning electron microscope (Jeol 35, Japan)[16].

Statistic analysis – most experiments were performed in duplicates, while SOD, ACE activity and methemoglobin concentration were

TABLE 1 Pre-radiation parameters of normal and abnormal red cells

	Control	Newborn	IDA
GSH	7.6 ± 0.4	8.7 ± 0.1***	11.8 ± 0.8****
(n)	(47)	(12)	(4)
MET	2.4 ± 0.1	3.2 ± 0.2****	3.9 ± 0.8*
(n)	(46)	(15)	(3)
SOD	2204 ± 121	2296 ± 69	2728 ± 184****
(n)	(51)	(12)	(3)
CAT	173 ± 10	123 ± 11****	183 ± 31
(n)	(52)	(15)	(5)
GP	6.8 ± 0.4	5.3 ± 0.4**	5.3 ± 1.1
(n)	(35)	(12)	(5)
GR	7.6 ± 0.4	8.5 ± 0.6	9.4 ± 0.6*
(n)	(52)	(15)	(5)
ACE	52.6 ± 2.4	34 ± 3.1****	74.3 ± 2.9****
(n)	(42)	(12)	(4)

Values are presented as means ± S.E
GSH levels are calculated as μmol/grHb.
Methemoglobin levels are given as percent (%) of total hemoglogin. GP, GR, CAT, and SOD activity in IU/grHb.
ACE is expressed in IU/ml cells.
*p<0.05
**p<0.025
*** p<0.01
**** p<0.005

performed in triplicates. Results are expressed as means ± S.E. The significance in data presented in Table 1 was determined by student's t-test. Calculations after radiation exposure were performed using Student's paired t-test. The advantage of the latter test is that each sample is used as its own control. This is necessary due to the great variance of individual data.

RESULTS

A. Pre-Radiation Levels: Enzymatic Activity, GSH and MET. Levels

Different parameters of fresh cells from newborn babies and IDA patients were determined and compared with healthy adults (blood units) (Table 1). Neonatal RBCs were found to have lower initial activities of CAT, 123 ± 11 vs 173 ± 10 IU/grHb, GP, 5.3 ± 0.4 vs 6.8 ± 0.4 IU/grHb and ACE levels of 34.3 ± 3.1 vs 52.6 ± 2.4 IU/ml cells. They had higher levels of MET, 3.2 ± 0.2 vs 2.4 ± 0.1% and GSH, 8.7 ± 1.0 vs 7.6 ± 0.4 μmol/grHb.

IDA RBCs were found to have higher initial levels of SOD, 2728 ± 184 vs 2204 ± 121 IU/grHb, GR activity of 9.4 ± 0.6 vs 7.6 ± 0.4 IU/grHb, ACE, 74.3 ± 2.9 vs 52.6 ± 2.4 IU/ml cells and GSH levels of 11.8 ± 0.8 vs 7.6 ± 0.4 μmol/grHb.

B. Exposure to Radiation

As irradiation of blood units (controls) at 1500 and 3000 cGy did not show any significant changes (data not shown), we irradiated fresh blood samples of IDA patients and placental blood as well as blood units, at 5000 cGy.

Even at the highest irradiation dose the activity of ACE, SOD, CAT, GP and GR did not show a significant change in the RBC from the different groups (Table 2). Neither did we find a change in GSH and methemoglobin levels (Table 3).

Potassium Leakage

Potassium concentrations in extracellular medium were measured before and after irradiation (Table 3). As expected, initial extracellular potassium levels were high in stored packed blood units, 3 to 10 days old: 22.7 ± 2.8 mM. Potassium levels increased insignificantly after exposure to

TABLE 2 Effect of radiation on RBC enzymes

		Control	Newborn	IDA
SOD	a	2448 ± 129	2296 ± 69	2728 ± 184
	b	2434 ± 11	2176 ± 69	2897 ± 229
CAT	a	171 ± 8	132 ± 11	184 ± 31
	b	169 ± 7	133 ± 10	166 ± 24
GP	a	5.8 ± 0.3	5.3 ± 0.4	5.3 ± 1.1
	b	6.2 ± 0.3	5.5 ± 0.4	5.1 ± 1.0
GR	a	7.4 ± 0.4	8.5 ± 0.6	9.4 ± 0.6
	b	7.3 ± 0.4	8.5 ± 0.6	9.1 ± 0.5
ACE	a	53.9 ± 2.2	34.0 ± 3.1	74.3 ± 2.9
	b	56.1 ± 2.2	34.3 ± 1.8	72.7 ± 6.1

Values are expressed as in Table 1.
a – Pre-radiation
b – Post-radiation

TABLE 3 Effect of radiation (5000 cGy) on RBC parameters

		Control	Newborn	IDA
MET (%)	a	2.1 ± 0.1	3.2 ± 0.2	3.9 ± 0.8
	b	2.1 ± 0.1	3.5 ± 0.2	4.0 ± 0.6
K^+ (mM)	a	22.7 ± 2.8	8.1 ± 0.8*	5.2 ± 0.6
	b	23.8 ± 3.3	8.9 ± 0.9*	5.8 ± 1.1
GSH μmol/grHb	a	8.2 ± 0.3	8.7 ± 1.0	11.8 ± 0.8
	b	8.0 ± 0.4	T9.3 ± 1.1	12.2 ± 0.8

Values are presented as means ± S.E
a – Pre-radiation
b – Post-radiation
* P = 0.002

5000 cGy to 23.8 ± 3.3. In IDA and neonatal cells there was a 10% increase in extracellular potassium levels. This increase was only significant in neonatal cells (P = 0.02), probably due to the largest number of blood samples.

C. Morphology

The morphology of the different cells was examined by scanning electron microscopy, before and after irradiation. Neonatal cells were heterogenous, with many stomatocytes. The stored cells consisted of discocytes and echinocytes, as expected. There were no morphological changes after radiation. (Data not shown.)

DISCUSSION

Radiation-induced oxidative damage in RBC is a result of the disturbed balance between its protective systems and the level of toxic oxygen products.[3] Activated oxygen species are produced during the normal metabolism of the cell, without apparent damage.[4] However, the cells are affected if their protective systems are malfunctioning or overwhelmed. GSH is known to protect the cells from the toxic effects of reactive oxygen compounds.[17] We have found GSH contents of RBC to be unaffected by the radiation doses used in our research (table 3), probably because the oxidative stress was not severe. The enzymatic activity of RBC antioxidant system, served as an indicator of activated oxygen species damage.[18] Previous studies have suggested a role for protein thiol groups in RBC radiation protection. Such thiol groups are present in GP and GR.[17] In addition, antioxidant enzymes have previously been found to undergo inactivation by free radicals such as peroxides and hydroxyl radicals.[19] Inactive forms of these enzymes are prone to proteolysis by macroproteinase, a proteolytic complex in RBC, which specifically interacts with oxidatively modified enzymes.[20] Therefore, a rapid rise in intracellular oxygen radicals, might partially destroy the protective systems. Finally, release of metals from metal containing proteins may further enhance oxidative stress by their role as catalysts of oxidation reduction reactions.[4,21,22] The *in vitro* activity of SOD, CAT, GR, or GP was not affected by radiation. These findings were consistent in all RBC types examined. Neonatal cells are known to have a significantly decreased optimal activity of NADH dep. MET Reductase. In addition, HbF has a higher affinity to oxygen and is more prone to oxidation than adult Hb, leading to specific hemoglobin oxidation product.[23,24] It is interesting to note that in neonatal and IDA red blood cells there is evidence of *in vivo* oxidative stress: their membranes have decreased amount of protecting thiol groups, altered lipid composition and a higher level of the lipid peroxidation product MDA.[24,25]

The RBC membrane, protected from oxidation by Vit E, cannot be effectively guarded by cytoplasmic antioxidant systems for steric reasons.[8]

Damage to protein or lipid components of the RBC membrane may lead to a leak of cytoplasmic components. Extensive damage might cause destruction of RBC membrane and lead to hemolysis.

We did not observe hemolysis or hematocrit change due to radiation damage in the different RBCs examined. Neither did we find a change in ACE optimal activity (Table 2). These observations, taken together with unchanged morphological data lead to the conclusion that extensive membrane damage did not occur. Yet, extracellular potassium levels appeared to increase in all cells irradiated by 5000 cGy. This increase was statistically significant only in neonatal cells, although in IDA cells there was the same trend of increase. It may be proposed that this occurs due to the inherent sensitivity in neonatal and IDA red blood cell membrane.[25] The initial high extracellular potassium in neonatal blood samples is most likely due to the handling of these sensitive cells prior to our experiment.

Therefore, we suggest that slight membrane damage did occur in all types of RBC irradiated at 5000 cGy. Our data support Brugnara's findings that radiation induces an increased permeability to potassium on the part of the RBC membrane.[26] Recent studies support high dose radiation (above 2500 cGy) to eliminate functional T lymphocytes and thus prevent TAGVHD[27]. This beneficial effect is mediated by chemical damage to cell constituents, mainly to the most sensitive target organ, the lymphocyte DNA. When transition metal ions are involved, $OH^{\bullet}$ radicals are liberated directly on the target molecule, thus sparing the membrane and intracellular protective systems.[28]

In summary, we conclude that radiation damage to RBC does occur as the $OH^{\bullet}$ radicals can react with other cellular components in addition to the target molecule. The damage to the membrane is ameliorated and therefore visible only under abnormal conditions, i.e. in newborn RBCs.

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