Dose-dependent survival of K562 cells subjected to irradiation, time course of endogenous enzyme activity and protective effect of applied superoxide dismutase

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Summary

Purpose: Irradiation-generated reactive species are proven to affect the cell survival and antioxidant enzyme levels. Radioresistance is a phenomenon which includes many cell mechanisms and signaling pathways. Superoxide dismutase (SOD) acts in and outside of cells after irradiation. The aim of this study was to determine LD₅₀ (lethal dose for 50% of K562 cells), to monitor the effect of a chosen dose and exogenously applied superoxide dismutase (ExSOD) on the cell number and the activity of SOD, glutathione peroxidase (GSH-Px) and catalase (CAT).

Methods: The survival of irradiated (20-32.5 Gy) K562 cells was determined using the trypan-blue exclusion. Besides irradiated and non-irradiated cells (controls), another two groups of cells were treated with SOD (10⁻⁶ M) which then served as SOD-treated controls or were irradiated (30 Gy) one hour later. The number of cells and the activity of SOD, GSH-Px and CAT (using kinetic methods) were monitored after 1, 24, 48, 72 and 96 hrs in unirradiated, irradiated, SOD-treated and SOD-treated/irradiated experimental groups.

Results: K562 cells showed dose-dependent survival in the chosen range of doses. A dose of 30 Gy induced 50% cell mortality and increased the activity of all three investigated enzymes after 24 hrs. Pretreatment with SOD preserved the survival of irradiated cells and increased SOD, GSH-Px and CAT activity. ExSOD induced an increase of the activity of all examined enzymes.

Conclusion: A balanced enhancement in endogenous antioxidative activity may be the cause of the increased radioresistance of K562 SOD-pretreated cells.

Key words: antioxidative enzymes, irradiation, K562 cells, radioprotection

Introduction

Oxidation, induced by reactive oxygen species (ROS), has long been known to play an important role in radiation effects and carcinogenesis as well [1-8].

Radioresistance of cells depends on its species, type of irradiation, delivered dose, different irradiation intervals and the kind of delivery (single or fractionated) [9,10]. Processes involved in radioresistance include various signal transduction pathways at nuclear and extranuclear levels [10-12]. Cell cycle arrest, induction of apoptosis and promotion of DNA-repair increase the radioresistance of cells subjected to irradiation in vitro and in vivo [13,14].

The role of endogenous antioxidative enzymes in defense from irradiation has been examined both in vivo and in vitro [6,7,15,16]. One important family of enzymes is the SOD [17,18]. They act inside and outside the cell, immediately after irradiation, and also in the post-irradiation
Beside their physiological generation inside the cell, superoxide radicals (O$_2^-$) are ensued at irradiation through water radiolysis. Superoxide is short-lived and does not readily cross bio-membranes, but it is converted through SOD to longer-lasting and membrane-diffusible hydrogen peroxide (H$_2$O$_2$) [22]. Hydrogen peroxide has broad regulatory and oxidative roles and can be further degraded to water by other antioxidant enzymes, such as GSH-Px and CAT [23]. It is supposed that H$_2$O$_2$ has a precise turnout in the cell differentiation through its activity in signal transduction [9,24].

Some investigators reported about overexpression of copper (Cu), and manganese (Mn) SOD after irradiation, but most consistently known consequence of x-ray application is elevation in MnSOD [22,25]. The removal of superoxide radicals by MnSOD in the mitochondria is a critical step in preventing radiation-induced cell death [26]. Overexpression of MnSOD blocks mitochondrial membrane permeability and the release of cytochrome C, probably by decreasing the superoxide radical level [27]. Also, IL-1 and TNFα have been noticed to activate MnSOD expression on RNA and protein level [28,29].

Studies on biological protection afforded by ExSOD included cell lines and experimental animals, as well as humans in clinical investigations [21,30-33]. Various strategies such as elevating the level of SOD inside and/or outside the cells have been employed [32,34]. ExSOD has beneficial effects in cases of postirradiation-induced dermatitis, fibrosis and diseases such as rheumatoid arthritis (RA), Crohn’s disease and progressive systemic sclerosis (PSS) [20,35].

In this study, we determined LD$_{50}$ of K562 cell line and examined the 96-hrs cell survival and the activity of three major antioxidative enzymes (SOD, GSH-Px and CAT) after irradiation with 30 Gy, as well as their activity when the cells were pretreated with SOD.

### Methods

The K562 cell line (human erythroleukemia) was routinely grown in 6-well tissue culture plates (Costar, USA) in RPMI 1640 medium (Sigma, New York, USA) supplemented with 2mM glutamine, 10% heat-inactivated foetal calf serum (NIVNS, Veterinary Institute, Novi Sad, Serbia), 100 IU/ml penicillin (ICN, USA) and 100 μg/ml streptomycin (ICN, USA). Cells were cultured at 37 °C in a humidified 5% carbon dioxide atmosphere. Exponentially growing K562 cells were used throughout the assays [15].

The cells were seeded in culture flasks at a number of 0.85x10$^6$/40 mL RPMI 1640 medium.

In the first part of the experiment, the cells were irradiated with 20-32.5 Gy, using a LINAC, Mevatron Siemens MD 7475, 10 MV X-rays, with a dose rate of 3 Gy/min and cultured up to 96 h after irradiation [15]. The trypan blue exclusion test (DET) was performed by mixing 50 μL of cell suspension with 200 μL of 0.1% trypan blue solution in 0.9% NaCl. After 2 min of incubation at room temperature, the number of viable (unstained) cells was determined using a Burker–Turk hemocytometer [36].

After selection of the irradiation dose (30 Gy), the samples were divided into four groups: 1. Control group (unirradiated samples); 2. Experimental group of unirradiated SOD-treated samples; 3. Experimental group of irradiated samples; and 4. Experimental group of irradiated and SOD-pretreated samples (Table 1).

For the antioxidant treatment of cells, the RPMI 1640 cell culture medium was supplemented with SOD (Peroxinorm CN953745 lot 17, Pliva, Zagreb, Croatia) at 10$^{-6}$ M concentration, and the incubation continued for one hour at 37°C.

The chosen experimental groups were irradiated with 30 Gy, as previously stated. The number of living cells was determined by DET according to Black, as previously described [36].

To obtain the cytosolic fraction, the cells were cen-
trifuged (10 min/1200 rpm), resuspended in saline and then frozen (–20°C) and thawed (37°C) for three times and finally centrifuged (10 min/10,000 rpm).

The protein concentration [37] and the activity of antioxidant enzymes SOD [38], GSH-Px [39], and CAT [40] were monitored in all experimental samples 1, 24, 48, 72 and 96 hrs post irradiation.

Statistics

All the experiments were performed in triplicate and enzyme analysis in quadruplicate. Results from a single experiment are shown and are representative of 2 further independent experiments. The results were expressed as mean±standard deviation of the mean (SDM).

The data were analyzed by multivariate analysis of variance (MANOVA) followed by Duncan’s test at 0.05 significance level to compare the means using the SPSS 13.0 for Windows.

Results

The survival of K562 cells was strongly dependent on the experimental time and the applied irradiation doses (Figure 1). High survival at all applied doses was registered in the 1st and the 96th hrs after irradiation. In the 1st h of the experiment, irradiation had an insignificant influence on survival, and the same was true for the 96th h when the cells had fully recovered from irradiation effects. LD50 of K562 cells was achieved with 30 Gy 24 hrs after irradiation.

Cells incubated with ExSOD and irradiated with 30 Gy grew in a similar fashion with the control cells up to 72 hrs after initiation of the experiment (Figure 2). At 96 hrs, a greater number of ExSOD-treated cells was found to survive when compared with untreated cells (Figure 2). The cells, preincubated with ExSOD prior to irradiation, showed increased survival at 24 and 48 hrs postirradiation when compared to irradiated cells (Figure 2). However, this was not seen at 72 and 96 hrs (Figure 2).

The activity of total endogenous SOD was elevated at all time points when cells were treated with ExSOD (Figure 3). This observation was also true for cells that were preincubated with ExSOD prior to irradiation (Figure 3). In the absence of ExSOD treatment, total endogenous SOD activity was increased only at 24 hrs postirradiation, but its activity decreased at later time points (Figure 3).

In the control, non-irradiated samples, changes in the GSH-Px activity were similar to those previously observed. Only at 24 hrs after irradiation, an increase in the activity of GSH-Px was observed (Figure 4). In cells treated with ExSOD, a substantial increase in GSH-Px activity was shown at 96 hrs (Figure 4).
Postirradiation K562 cell survival with superoxide dismutase administration

Figure 2. Long-term survival of K562 cells subjected to irradiation, incubated with SOD or a combination of both treatments. U: unirradiated cells; U+SOD: unirradiated cells pretreated with SOD; I: irradiated cells; I+SOD: irradiated cells pretreated with SOD. Results from a single experiment are shown and are representative of two further independent experiments. Means pairs followed by different letters are significantly different (p<0.05) by Duncan’s test, n = 4. Legend: a) U↔U+SOD; b) U↔I; c) U↔I+SOD; d) U+SOD; e) U+SOD↔I+SOD; f) I↔I+SOD.

Figure 3. Time course of total endogenous SOD activity in K562 cells subjected to irradiation, incubated with SOD or a combination of both treatments. U: unirradiated cells; U+SOD: unirradiated cells pretreated with SOD; I: irradiated cells; I+SOD: irradiated cells pretreated with SOD. Results from a single experiment are shown and are representative of two further independent experiments. Means pairs followed by different letters are significantly different (p<0.05) by Duncan’s test, n = 4. Legend: a) U↔U+SOD; b) U↔I; c) U↔I+SOD; d) U+SOD; e) U+SOD↔I+SOD; f) I↔I+SOD.
Figure 4. Time course of endogenous GSH-Px activity in K562 cells subjected to irradiation, incubated with SOD or a combination of both treatments. U: unirradiated cells; U+SOD: unirradiated cells pretreated with SOD; I: irradiated cells; I+SOD: irradiated cells pretreated with SOD. Results from a single experiment are shown and are representative of two further independent experiments. Means pairs followed by different letters are significantly different (p<0.05) by Duncan’s test, n=4. Legend: a) U↔U+SOD; b) U↔I; c) U↔I+SOD; d) U+SOD; e) U+SOD↔I+SOD; f) I↔I+SOD.

Figure 5. Time course of endogenous CAT activity in K562 cells subjected to irradiation, incubated with SOD or a combination of both treatments. U: unirradiated cells; U+SOD: unirradiated cells pretreated with SOD; I: irradiated cells; I+SOD: irradiated cells pretreated with SOD. Results from a single experiment are shown and are representative of two further independent experiments. Means pairs followed by different letters are significantly different (p<0.05) by Duncan’s test, n=4. Legend: a) U↔U+SOD; b) U↔I; c) U↔I+SOD; d) U+SOD; e) U+SOD↔I+SOD; f) I↔I+SOD.
CAT activity showed a similar trend when compared to the activity of GSH-Px. A substantial increase in CAT activity was only found 24 h postirradiation (Figure 5). CAT activity was greater in SOD-treated cells than in untreated cells at 48, 72 and 96 hrs.

Discussion

It is well known that leukemic cells have a higher vulnerability to ionizing radiation than normal mononuclear cells [9]. On the contrary, some authors report very potent radiosensitivity of K562 cells [41,42]. In the present investigation, K562 cells showed significant resistance to irradiation in a dose-dependent manner, with a LD$_{50}$ of 30 Gy 24 h postirradiation.

The basic low SOD activity and overproduction of O$_2$. In cancer cells may render them highly dependent on SOD for survival [43]. Exogenous sources of superoxide generation, such as irradiation, have additional deleterious effects. Irradiation-caused inhibition of SOD leads to free radical damage of membranes and apoptosis of cancer cells [45]. In our experiment, the number of K562 cells irradiated with 30 Gy was reduced to 50% after 24 hrs. Later on, recovery of the number of surviving cells was noticed. Twenty-four hrs postirradiation was the critical time point for finishing of the apoptotic processes, as well as for enhancement of the enzyme activity. After the initial decrease due to irradiation, all of three major enzymes were strongly activated in the survived cell population to defend cells from oxidative damage. We registered a significant increase in SOD, GSH-Px and CAT activity 24 hrs after irradiation. This is in correlation with investigations which confirmed that antioxidant mechanisms are activated around 24hrs after irradiation, thus eliminating the levels of ROS by metabolizing them to neutral products [21,44].

Exogenously added SOD does not penetrate readily into the cells, but it may scavenge superoxide radicals outside the cell and therefore serves as a first line of defense against irradiation [5]. In the experiment, the added Ex-SOD was supposed to serve as a scavenger of exogenous superoxide radicals and producer of H$_2$O$_2$. In SOD-pretreated and irradiated group, the results implied that the survival in this group was at the same level as in the control group during the whole experiment.

Also, it was noticed in our study that preincubation with SOD protects cells from injury immediately after irradiation. In the 1st h after the X-ray delivery, there was a significant increase in the activity of all monitored enzymes in the groups pretreated with SOD in comparison to the irradiated groups. These results support the hypothesis that SOD may act around and within the irradiated cells by scavenging the short-lived superoxide radicals produced at the time of irradiation, as well as those produced as a result of oxidative metabolism after the exposure.

Furthermore, accumulating evidence suggests that reactive oxygen species (ROS) are not only injurious byproducts of cellular aerobic metabolism but also essential participants in cell signaling and regulation. MnSOD, which may be induced by free radical challenge, is synthesized in the cytosol and post-translationally modified for transport into the mitochondria [45]. In the cells exposed to adverse conditions, increased activity of MnSOD is a survival factor that holds mitochondrial integrity [3,46]. Increased MnSOD increases the concentration of endogenously produced H$_2$O$_2$. At 24th h postirradiation, the intracellular production of H$_2$O$_2$ was a secondary event [21]. It was shown that intracellular H$_2$O$_2$ is involved in enhancing/modulating the activity of NF-kB in a cell type-specific manner [47]. The response of NF-kB to H$_2$O$_2$ depends on time, concentration, and the regime of exposure [48]. Thus, it seems that exogenous SOD produces H$_2$O$_2$, which, according to Adzic et al., induces MnSOD expression and increases the intracellular H$_2$O$_2$ production, triggers NF-kB thereby forming a positive feed-forward loop changing the redox potential towards oxidation [49].

It is well known that small changes in the redox potential towards oxidation may significantly elevate the proliferative capacity of malignant cells [50]. In response to that schedule, an increase in the antioxidative enzymes SOD, GSH-Px and CAT activities led to restore a reduced state of the cell. Together with SOD and GSH-Px, CAT constitutes the primary defense against ROS and may provide resistance to the effects of chemotherapy and ionizing radiation. The obtained results were in agreement with these findings.

In conclusion, it may be reported that K562 cells show significant resistance to irradiation in a dose-dependent manner. Irradiation at the applied dose of 30 Gy has a strong effect on survival with LD$_{50}$ 24 hrs postirradiation. After the primary inhibition due to irradiation, all of the three major enzymes are activated together to repair cells from oxidative injury. The results indicate that pretreatment with SOD protects cells from irradiation-induced apoptosis.
However, because the balance of the enzymes is of utmost importance for oxidative protection, every impact on this equilibrium-in this case in excess of SOD in the absence of irradiation-may lead to misbalance, which should change the response in terms of sensitivity to oxidative stress. This condition might make cells vulnerable to further injury.

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References


