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Communication

Labile iron pool correlates with iron content in the nucleus and the formation of oxidative DNA damage in mouse lymphoma L5178Y cell lines★✪

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Labile iron pool (LIP) constitutes a crossroad of metabolic pathways of iron-containing compounds and is midway between the cellular need for iron, its uptake and storage. In this study we investigated oxidative DNA damage in relation to the labile iron pool in a pair of mouse lymphoma L5178Y (LY) sublines (LY-R and LY-S) differing in sensitivity to hydrogen peroxide. The LY-R cells, which are hydrogen peroxide-sensitive, contain 3 times more labile iron than the hydrogen peroxide-resistant LY-S cells.

Using the comet assay, we compared total DNA breakage in the studied cell lines treated with hydrogen peroxide (25 μM for 30 min at 4°C). More DNA damage was found in LY-R cells than in LY-S cells. We also compared the levels of DNA lesions sensitive to specific DNA repair enzymes in both cell lines treated with H_2O_2. The levels of endonuclease III-sensitive sites and Fapy-DNA glycosylase-sensitive sites were found to be higher in LY-R cells than in LY-S cells.

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Abbreviations: LIP, labile iron pool; Fapy-DNA glycosylase-DNA glycohydrolase [2,6-diamino-4-hydroxy-5-(*N*-methyl)formamidopyrimidine releasing] (EC 3.2.2.23); endonuclease III, DNA-(apurinic or apyrmidinic site) 5′-phosphomonoester-lyase (EC 4.2.99.18).

Our data suggest that the sensitivity of LY-R cells to H_2O_2 is partially caused by the higher yield of oxidative DNA damage, as compared to that in LY-S cells. The critical factor appears to be the availability of transition metal ions that take part in the OH radical-generating Fenton reaction (very likely in the form of LIP).

The unique abilities of iron to change its oxidation state and redox potential in response to changes of liganding environment enable it to play an essential role in many biological reactions. However, the same physiochemical properties that make iron essential for most living organisms also make it very dangerous. Trace amounts of "free" iron may catalyze generation of a highly toxic hydroxyl radical *via* Fenton/Haber–Weiss reaction cycle. Living organisms try to avoid an excess of "free" iron by tightly controling iron homeostasis. In most cells iron homeostasis consists of iron uptake, utilization and storage. The principal effectors of these processes are transferrin receptor, a protein involved in iron uptake, and ferritin, an iron-sequestering protein. Since uptake and storage of iron is carried out by different proteins, there is a pool of accessible iron ions, called labile iron pool (LIP), that constitutes crossroads of the metabolic pathways of iron containing compounds. LIP is a low-molecular-mass pool of weakly chelated iron that rapidly passes through the cell. LIP level is midway between the cellular need for iron and the hazard of excessive generation of hydroxyl radical, as it has been proposed that LIP is a cellular source of iron ions available for Fenton reaction (Breuer *et al.*, 1997).

Mouse lymphoma L5178Y-R (LY-R) and L5178Y-S (LY-S) cell lines are inversely cross-sensitive to hydrogen peroxide and ionizing radiation. A spontaneous loss of radiation resistance of LY-R cells and its conversion into radiation-sensitive LY-S cells was accompanied by an unexpected increase in H_2O_2 resistance of the latter cells (Beer *et al.*, 1983; Bouzyk *et al.*, 1991; Kruszewski & Szumiel, 1994). The lower H_2O_2 sensitivity of LY-S cells than that of LY-R cells is related to their better antioxidant defence system (Bouzyk *et al.*, 1997) and lower content of transition metal ions in the nucleus (Szumiel *et al.*, 1995). In this study, we used a modified comet assay to assess the oxidative DNA damage inflicted in both cell lines by H_2O_2 treatment.

MATERIALS AND METHODS

Cell cultures and H_2O_2 treatment. Murine lymphoma cells L5178Y-R (LY-R) and L5178Y-S (LY-S) were kept in suspension culture in Fischer's medium, as described in (Szumiel, 1979). Exponentially growing cell cultures were centrifuged, cells were harvested and resuspended in ice-cold phosphate-buffered saline (PBS) without calcium and magnesium (PBS(–)) to the final density of 400 000 cell/ml. Appropriate aliquot of H_2O_2 was added to the cell suspension to the final concentration 25 μM and cells were incubated for 1 h in ice-bath. After the incubation the cells were harvested by centrifugation, resuspended in ice-cold PBS(–) and processed according to the comet assay section.

Comet assay. The initial DNA damage was determined by the alkaline version of the 'comet' assay, performed as described by Kruszewski *et al.* (1998). Briefly, approximately 0.5 ml of cell suspension was mixed with low melting point agarose and cast on a microscope slide. After lysis the slides were left in fresh electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH, pH >13) in order to allow DNA unwinding and electrophoresed (1.2 V/cm, 30 min, 10°C). Pictures of 100 randomly selected comets per slide were captured. Image analysis of the data was by the Comet v.3.0 (Kinetic Imaging Ltd., Liverpool, U.K.).

For nuclease sensitive sites estimation the procedure was similar, except that after lysis the slides were washed 3× in glycosylase buffer (40 mM Hepes/KOH, 0.1 M KCl, 0.5

mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0). The slides were then treated with either glycosylase in the buffer or buffer alone. Further steps were the same as for DNA breaks estimation. Enzyme treated slides and buffer controls were electrophoresed concurrently.

RESULTS AND DISCUSSION

Despite the essential role of iron as a cofactor of many biochemical reactions, its abundance in living cells is potentially hazardous. In the presence of superoxide anion radical and hydrogen peroxide, iron can catalyze generation of the very reactive hydroxyl radical ($OH^{\bullet}$). Interaction of $OH^{\bullet}$ with cellular components may result in damage to biomolecules, i.e. DNA, RNA or proteins. Although the amount of redox-active iron is tightly controlled, an involvement of iron in the induction of oxidative damage to the biomolecules is well documented (Henle *et al.*, 1996; Meneghini, 1997; McCord, 1998; Touati, 2000; Emerit *et al.*, 2001).

LIP level in L5178Y cells is associated with iron content in the nucleus

All aspects of intracellular iron homeostasis are mirrored in the LIP level, weakly chelated iron that rapidly passes through the cell. Since a fluorimetric assay to assess the LIP level in intact living cells has recently become available (Epsztejn *et al.*, 1997), we measured LIP in L5178Y cell lines. The LIP level in LY-R cells is 3-times higher than in LY-S cells (Lipinski *et al.*, 2000) (Table 1). Interestingly, it is well correlated with the content of iron in L5178Y cell nuclei, previously measured by flame atomic absorption spectrometry (Szumiel *et al.*, 1995) (Table 1).

Little is known about labile iron content and distribution in living cells. Although numerous methods for its characterization have been developed during the last decades, most of these methods are not applicable to viable biological material. Measurements of total cellular iron do not reflect the actual abundance of iron ions available for redox reactions, as most of cellular iron is bound to heme and non-heme proteins and does not enter Fenton reaction. Recently, fluorescence spectroscopic methods for measurements within viable cells have become available. Using laser scanning microscopy Petrat *et al.* (2001) were able to determine the concentration of chelatable iron and study its intracellular distribution. They found that in isolated rat hepatocytes LIP concentration is 5.8 ± 2.6 μM. These data are slightly higher than those obtained by us and others. This discrepancy might be due to the use of different cell lines (hepatocytes *versus* lymphoblasts), fluorescent probes and chelators. However, our results are in good agreement with the data obtained by J. Cabantchik and colleagues in human erythroleukaemia K562 cells with the same fluorescent probe and chelator (Epsztejn *et al.*, 1997; Breuer *et al.*, 1996; Konijn *et al.*, 1999).

Interestingly, Petrat *et al.* (2001) found that hepatocyte nucleus contained 6.6 ± 2.9 μM of

Table 1. Labile iron pool and iron content in the nuclei of L5178Y cell lines

	L5178Y-R (a)	L5178Y-S (b)	a/b
H_2O_2-sensitivity	High	Low	
Total LIP [μM] (Lipiński *et al.*, 2000)	0.57 ± 0.19	0.18 ± 0.8	3.16
Total Fe content in the nucleus (ng/10^6 cells) (Szumiel *et al.*, 1995)	7.7 ± 1.8	3.1 ± 0.9	2.48

chelatable iron. Although the amount of total concentration iron in hepatocytes was estimated to be similar to that of the cytoplasm (Lai *et al.*, 1996), a surprisingly high content of redox-active iron in the nucleus presents a hazard of DNA damage.

Oxidative DNA damage in L5178Y cell lines differing in LIP level

Mouse lymphoma LY-R and LY-S cell lines are exceptional among mammalian cellular models due to their unique inverse cross-sensitivity to hydrogen peroxide and ionizing radiation. In this study, we investigated whether the difference in the cytosolic LIP level found in this pair of cell lines is reflected in the difference in induction of oxidative DNA damage by hydrogen peroxide. Figure 1 shows the total DNA breakage (sum of single + double strand breaks and alkali labile

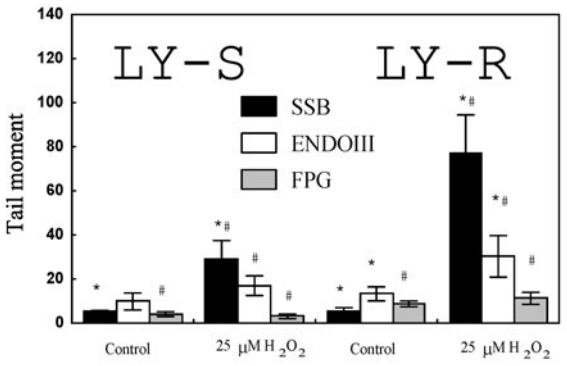

Figure 1. H_2O_2 induced DNA damage in LY cell.

*Statistically significant difference treated *vs* control, $P < 0.05$; #statistically significant difference LY-R *vs* LY-S, $P < 0.05$.

sites) as well as endonuclease III- and Fapy-DNA glycosylase-sensitive sites in LY cells treated with 25 μM hydrogen peroxide. In control cells, the level of strand breaks and endonuclease III-sensitive sites was similar in both cell lines, however, we found a statistically significant difference ($P < 0.05$) in the level of Fapy-DNA glycosylase-sensitive sites. In LY-S cells treated with 25 μM hydrogen peroxide, only the level of the total DNA breakage significantly differed from the control cells. In contrast, in LY-R cells treated with H_2O_2 a significant difference from the control was found in the case of total DNA breakage and endonuclease III-sensitive sites. In the treated cells the yield of DNA breaks and endonuclease III- and Fapy-DNA glycosylase-sensitive sites were all significantly higher in LY-R cells than in LY-S cells. Interestingly, neither in LY-R nor LY-S cells did we find any significant difference in the yield of Fapy-DNA glycosylase-sensitive sites between control and treated cells.

Iron-driven Haber–Weiss reaction gives rise to very toxic reactive oxygen species ($OH^{\bullet}$ in particular). Thus, iron storage proteins must guard their iron in a form that does not undergo Haber–Weiss reaction. On the other hand, there is a physiological demand for easily accessible iron that can be incorporated to numerous iron containing proteins. A critical point in understanding the mechanism of iron homeostasis in mammalian cells was the practical demonstration of the existence of a transient pool of weakly chelated iron, now referred to as LIP (Epsztejn *et al.*, 1997). The role of LIP in the induction of oxidative DNA damage was unclear, until the demonstration by Petrat *et al.* (2001) of the existence of a chelatable iron pool in the cell nucleus. It was plausible to assume that this nuclear redox-active iron pool may be involved in DNA damage induction by hydrogen peroxide and other oxidizing compounds. Indeed, a significant correlation between LIP level and the yield of 8-oxo-7,8-dihydro-2′-deoxyguanosine, a typical marker of reactive oxygen species-induced DNA damage, was found in human lymphocytes (Gackowski *et al.*, 2002).

Here we demonstrated that the level of cytosolic LIP in LY cells is associated with the yield of DNA damage induced by H_2O_2. In the LIP-rich LY-R cells the yield of DNA breaks, endonuclease III- and Fapy-DNA glycosylase-sensitive sites was significantly higher than in the low-LIP LY-S cells. These data con-

firm our previous findings that the lower H_2O_2 sensitivity of LY-S cells is accompanied by a lower initial damage of DNA bases generated by H_2O_2 treatment, as compared with LY-R cells (Zastawny *et al.*, 1998). A correlation between cellular sensitivity to DNA damaging agents and the yield of DNA base damage was demonstrated for the first time by Mori and Dizdaroglu for the radiation-sensitive M10 mutant of the L5178Y cell line (Mori & Dizdaroglu, 1994). However, gas chromatography/mass spectrometry used in those studies required very high doses of DNA damaging agents to get reproducible results. Here we were able to confirm our previous results with a more sensitive and reliable method.

Our data suggest that the sensitivity of LY-R cells to H_2O_2 is partly caused by a higher yield of oxidative DNA damage, as compared to that in LY-S cells. Although other factors, such as differences in antioxidant defence (Bouzyk *et al.*, 1997), cannot be excluded, a critical factor in the cause–effect relation appears to be the availability of transition metal ions that take part in the OH radical-generating Fenton reaction (very likely in the form of LIP).

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