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Induction of DNA breakage in X-irradiated nucleoids selectively stripped of nuclear proteins in two mouse lymphoma cell lines differing in radiosensitivity

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The role of nuclear proteins in protection of DNA against ionizing radiation and their contribution to the radiation sensitivity was examined by an alkaline version of comet assay in two L5178Y (LY) mouse lymphoma cell lines differing in sensitivity to ionizing radiation. LY-S cells are twice more sensitive to ionizing radiation than LY-R cells (D_0 values of survival curves are 0.5 Gy and 1 Gy, respectively). Sequential removal of nuclear proteins by extraction with NaCl of different concentrations increased the X-ray induced DNA damage in LY-R nucleoids. In contrast, in the radiation sensitive LY-S cell line, depletion of nuclear proteins practically did not affect DNA damage. Although there is no doubt that the main cause of LY-S cells' sensitivity to ionizing radiation is a defect in the repair of double-strand breaks, our data support the concept that nuclear matrix organisation may contribute to the cellular susceptibility to DNA damaging agents.

DNA in the nucleus of eukaryotic cells is tightly associated with histones and other nuclear proteins and folded into a higher order chromatin structure that is anchored to the nuclear matrix. Chromatin proteins are important not only for the maintenance of chromatin structure but also may protect DNA against exogenous damage. Regions of chromatin of more open conformation, like transcriptionally active DNA, are more susceptible to ionizing radiation than inactive regions [1, 2]. Proteins may also protect DNA against

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Abbreviations: DPC's, DNA-protein crosslinks; DSB's, double-strand breaks; NHP's, non-histone proteins; DAPI, 4′,6-diamidino-2-phenylindole; D_0, the dose required to reduce the surviving fraction by the factor e^{-1}.

radiation-generated free radicals, or may serve as a source of reducing equivalents for chemical repair of DNA radicals.

To examine the role of nuclear proteins in protection of DNA against ionizing radiation and their contribution to the radiation sensitivity, an alkaline version of comet assay, which detects overall DNA damage i.e. DNA breaks and alkali-labile sites, was used to estimate DNA damage [3].

MATERIALS AND METHODS

Cells of interest were embedded in low melting point agarose and pipetted on microscope slides. After solidification the slides were placed in lysis solution (100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10) and held at 4°C for 30 min to produce nucleoids. Slides were then rinsed with lysis solution without Triton X-100 and maintained overnight in this solution without Triton X-100, containing 0.14 M, 0.8 M or 2.5 M NaCl. Using different NaCl concentrations we can manipulate the protein content of the nucleoids. Treatment with 0.14 M NaCl removed a small portion, if any, of non-histone proteins (NHP's); 0.8 M NaCl removed the majority of histone H1 and NHP's. Extraction with 2.5 M NaCl removed all histone proteins and the majority of NHP's [4, 5]. After NaCl extraction the slides were rinsed and irradiated when kept on ice with a dose of 1.5 Gy of X-rays (X-ray machine Stabilipan, Siemens, Germany). After irradiation the slides were carefully submerged in lysis solution with Triton X-100 and left for 1 h at 4°C to remove all remaining proteins. The slides were then transferred into an electrophoretic tank, left for 40 min at 4°C in the electrophoretic buffer (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for unwinding and electrophoresed (30 min, 25 V, 1 V/cm). After electrophoresis the slides were washed three times with an excess of 0.4 M Tris (pH 7.5) and stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/ml in water). DNA damage in individual cells was quantified by means of computer supported image analysis system Comet v.3.0 (Kinetic Imaging Ltd., Liverpool, U.K.). Under the same electrophoretic conditions the extent of DNA migration should be correlated with the DNA damage (unless DNA crosslinks are involved). The percentage of DNA in the comet "tail" was used as a measure of DNA damage.

RESULTS AND DISCUSSION

The cellular model used in this study consists of two L5178Y (LY) mouse lymphoma cell lines, LY-S and LY-R, differing in sensitivity to ionizing radiation; D_0 values of survival curves (the dose required to reduce the surviving fraction by the factor e^{-1}) are 0.5 Gy and 1 Gy, respectively. In control (non-irradiated) cells the sequential selective removal of chromatin proteins from the nucleoids did not result in any significant changes in DNA mobility (Table 1). However, we found significantly more DNA in the comet tail of control LY-R cells than in LY-S cells ($P < 0.05$). This phenomenon may reflect a higher level of endogenously generated DNA damage in LY-R cells due to the higher steady-state transition metal ion content (for discussion see [6]) and has repeatedly been observed by us in the comet assay with LY cells. Another explanation of this phenomenon may be a different chromatin organisation in LY sublines, previously proposed in view of the different supercoiling properties of nucleoids from LY cells [7]. Nevertheless, the initial DNA damage induced in unextracted chromatin with 1.5 Gy of X-ray radiation was similar in the two cell lines (Table 1). A similar level of initial DNA breakage was also found previously in non-exposed cells [8].

Sequential removal of nuclear proteins resulted in an increase of DNA damage in LY-R nucleoids. Removal of histone H1 and a part of NHP's (0.8 M NaCl) caused 1.10-times increase in the mean DNA content in the comet

Table 1. X-ray radiation induced DNA breakage in nucleoids of L5178Y sublines extracted with different salt concentrations

		Percentage of DNA in the comet tail	
	NaCl [M]	L5178Y-R	L5178Y-S
Control	0.14	12.3 ± 6.8[b]	7.1 ± 5.5[b]
	0.80	14.3 ± 7.9[b]	7.2 ± 4.8[b]
	2.50	13.9 ± 6.5[b]	6.4 ± 3.9[b]
X-irradiated 1.5 Gy	0.14	32.7 ± 7.2[a,d,e]	30.2 ± 8.6[a]
	0.80	38.3 ± 8.2[a,b,c]	31.5 ± 9.2[a,b]
	2.50	41.0 ± 10.6[a,b,c]	29.8 ± 7.9[a,b]

Mean ±S.D. of 50 comets. Student's *t*-test was used to evaluate significance of the difference between means, $P < 0.05$; [a]significant difference *vs.* control; [b]significant difference *vs.* another LY subline; [c]significant difference *vs.* 0.14 M NaCl; [d]significant difference *vs.* 0.80 M NaCl; [e]significant difference *vs.* 2.50 M NaCl.

tails of the irradiated nucleoids, as compared to nucleoids treated with 0.14 M NaCl ($P < 0.01$). Total removal of histones and NHP's (2.5 M NaCl) resulted in 1.25-times increase in DNA damage of irradiated nucleoids ($P < 0.01$, Fig. 1). In contrast, in the radiation sensitive LY-S cell line, depletion of nuclear protein practically did not affect DNA damage (Fig. 1).

The protective role of nuclear proteins is well established. Although histone proteins do not always protect DNA against damaging agents [9], their removal from DNA prior to irradiation results in a significant increase in DNA damage [4, 5, 10–13]. Elia & Bradley [10] and Olive & Banath [5] observed an about 20-fold increase in the number of radiation-induced double strand breaks (DSB's) in isolated nucleoids pre-treated with 2 M NaCl. A much higher level of damage (about 100-fold) was observed in histone depleted nuclei examined using the alkaline unwinding method [11, 12] as compared to irradiated intact cells. However, only 1.6-times increase in percentage of DNA in the comet tail was found when irradiated nucleoids extracted with 0.14 M NaCl and 2 M NaCl were compared [5].

Our results indicate that extractable nuclear proteins play an important role in maintaining DNA integrity in LY-R cells; in contrast, their removal from nucleoids of LY-S does not change chromatin susceptibility to ionizing radiation. The level of DNA breakage in non-extracted chromatin is higher in LY-S than in LY-R cells (after control values subtraction, Fig. 1) indicating that either protection of LY-R nucleoids by nuclear proteins is better, or there are more DNA-protein crosslinks (DPC's) in LY-R cells. However, the latter is not the case, as in both cell lines the yield of radiation-induced DPC's is similar (I. Grądzka, unpublished). Our results are in

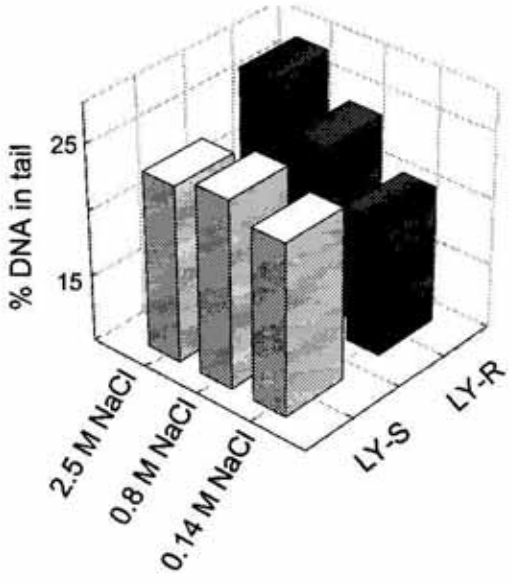

Figure 1. DNA damage in protein stripped nucleoids of L5178Y cell lines expressed in percent of DNA in the comet "tail".

Data represent mean values for 50 comets after subtraction of mean values for 50 control comets.

agreement with the earlier report by Kapiszewska *et al.* [14], in which differences in nuclear matrix composition were claimed to be responsible for different rewinding properties of DNA in LY-R and LY-S nucleoids. The authors also found that it was a lack of nuclear protein of about 55 kDa in LY-S cells that could have affected nuclear matrix properties. Although there is no doubt that the main cause of LY-S cells' sensitivity to ionizing radiation is a defect in the repair of DSB's, our data support the concept that nuclear matrix organisation may contribute to the cellular susceptibility to DNA damaging agents, as suggested by Roti Roti *et al.* [15].

Those authors found a relationship between radiation sensitivity and the absence or reduced abundance of some nuclear matrix proteins in cellular models that comprised L5178Y variants (radioresistant and radiosensitive), Chinese hamster mutants, and rat embryo cells transfected with oncogenes (*H-ras* and *c-myc*) that increased radioresistance [16-18].

The proposed relation between nuclear matrix proteins, stability of supercoiling and radiosensitivity may involve damage fixation. A DSB's-containing DNA domain that undergoes a supercoiling change before the DSB's can be repaired would cause its fixation. Thus the DSB's would be left unrepaired and hence, lethal. The presented data are consistent with such a conclusion.

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