DNA Supercoiling and Repair in Peripheral Lymphocytes as a Measure of Acute Radiation Response After Radiotherapy

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DNA Supercoiling and Repair in Peripheral Lymphocytes as a Measure of Acute Radiation Response After Radiotherapy

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SUMMARY DNA supercoiling density and incision kinetics during ultraviolet (UV) excision repair hav been measured in lymphocytes from 20 cancer patients and 17 healthy donors. Nucleoid sedimentation was used, which allows the sensitive detection of both DNA damage and alterations in chromatin structure. The release of DNA supercoiling after ethidium bromide intercalation and the kinetics of the incision step following UV irradiation were compared in lymphocytes derived from cancer patients and those from normal donors. The classification into lymphocytes with normal or reduced repair and normal or altered supercoiling, respectively, revealed that reduced repair as well as altered chromatin structure occurred more frequently in lymphocytes derived from patients (40% and 85%, respectively) than in those from healthy donors (35% and 23%, respectively). Even more striking was the simultaneous occurrence of both characteristics in tumor patients: in 34% of all cases reduced repair was associated with altered supercoiling density, whereas among healthy donors this association occurred in only 18% of all cases. Supercoiling density may be related to functional integrity of lymphocytes and repair capacity to recovery after radiation damage. Since both parameters are important for the radiation response of normal tissue, we consider these measurements a potential prognostic assay aimed at reducing acute reaction of the normal tissue. *Radiat Oncol Invest 1994;2:126–133.*

Key words: lymphocytes, chromatin structure, DNA repair, cancer patients

INTRODUCTION

The detection of genetic defects that cause enhanced radiosensitivity of normal tissue is of great importance for further progress in radiotherapy (RT). It has been estimated that the exclusion of only 5% of the most sensitive patients from RT may lead to a considerable increase in the percentage of local control [1].

Damage to lymphocytes may be one cause of acute radiation reactions and thus may represent a limiting factor in RT. On the other hand, the concept of patient-specific inherent radiation sensitivities may allow one to extrapolate from the radiation response of lymphocytes to estimate the radiosensitivity of other tissues. There is a very limited number of studies comparing the radiosensitivity of pe-

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Robert Roessle Clinic/Center Institute for Cancer Research is now part of the Max Delbrueck Center for Molecular Medicine, Berlin-Buch, Germany.

ripheral lymphocytes with that of other normal tissues. Green et al. [2] did not find any correlation comparing the survival after gamma-irradiation in T-lymphocytes and skin fibroblasts of 33 healthy donors. On the other hand, radiation hypersensitivity of ataxia-telangiectasia (AT) individuals or AT heterozygotes is well reflected by their lymphocytes [3], indicating that an inherent radiosensitivity that falls out of the normal range due to a genetic defect can also be detected in lymphocytes. Similar findings have been reported for lymphoblastoid cells from Gardner's syndrome patients [4]. It may thus be useful to measure lymphocyte radiosensitivity in order to tailor an optimal radiation scheme for the individual patient and to minimize radiation damage to normal tissue.

One of the most sensitive targets for radiation damage in mammalian cells is higher order DNA structure. Changes in supercoiling status can be measured after very low radiation doses [5,6]. The supercoiled structure of chromosomal DNA seems to be a general feature for spatial organization of chromatin and mediates regulation of enzymatic processes on the DNA of eukaryotic cells [7,8]. Several studies carried out on normal cells as well as on tumor cell lines indicate a relation between the stability of supercoiled DNA and cellular radiosensitivity [9,10].

It has been shown that DNA organization differs between transcriptionally active and inactive gene regions [11–13]. These findings allow one to assume that any exogenous disturbance of supercoiled structure may have an influence on gene expression in the related chromatin region. This could be related to the recovery of the immune response of the hematopoietic system after irradiation. In human lymphocytes the age-related decrease of the repair efficiency for ultraviolet (UV)-induced DNA damage is associated with an increase in supercoiling density [14], indicating a relation between chromatin organization and repairability of DNA damage.

In this study we investigated DNA supercoiling density and repair kinetics for UV-induced base dimers in lymphocytes from cancer patients and healthy donors using the nucleoid sedimentation technique. This method allows both the detection of supercoiled relaxation caused by an intercalating dye as well as the time course of the incision step, the first enzymatic reaction of DNA excision repair. Furthermore, this method has the advantage that it avoids any stimulation of the lymphocytes, thus studying their radiation response in the normal G_0 phase.

MATERIALS AND METHODS

Origin and Preparation of Lymphocytes

The cancer patients included in this study were diagnosed between January and October 1991 at the Robert Roessle Clinic/Central Institute for Cancer Research (Berlin) with stomach or colon cancer. All studies have been carried out prior to any treatment. All the patients were between 50 and 73 years old, with 85% between the age of 56 and 70 years. A group of healthy volunteers was chosen with a comparable age distribution to provide control lymphocytes.

Ten milliliters of venous blood was taken from the donor and held in sodium citrate-coated tubes. The lymphocytes were isolated using Ficoll™ sedimentation. This yielded 95% lymphocytes and 5% monocytes. After washing 3× in phosphate buffered saline (PBS), the lymphocytes were spun down and suspended in RPMI medium containing 10% fetal calf serum (FCS; local supplier), 100 IU/ml penicillin sodium salt (Jenapharm GmbH, Jena, Germany), and 100 μg/ml streptomycin (Jenapharm GmbH, Jena, Germany). Experiments were carried out following 12 hr incubation at 37°C (concentration $5 \cdot 10^5$ cells/ml). Trypan blue exclusion detected less than 5% dead cells.

Measurement of the DNA Supercoiling Density

Nucleoid sedimentation was carried out as described previously by Cook and Brazell [15,16]. Briefly, a linear 5–20% sucrose gradient [plus the required ethidium bromide (EtBr) concentration] prepared in polyurethane ultracentrifugation tubes (10 mm diameter, 35 mm length, Beckmann Corp., Palo Alto, CA) was overlaid with 100 μl lysis solution (1 M NaCl, 100 mM EDTA, 1% Triton X-100, pH 7.2); 10^5 cells were carefully rinsed onto the top of the gradient and held for 20 min in the dark at 18°C. This procedure yields nuclei with removed histone proteins, so-called nucleoids, which preserve the supercoiled tension of the DNA due to its binding to the nuclear matrix [17]. The fluorescence dye EtBr (Sigma, St. Louis, MO) intercalates into the DNA strand, resulting in a concentration-dependent reduction (decreasing negative coiling) of the packing density of the nucleoids, followed by an increase at higher EtBr concentration (positive coiling). This mechanism is measured by running the samples on an ultracentrifuge (Beckmann L5, SW50 Rotor), operating at 20,000 rpm for 30 min. The sedimentation distance is measured relative to the top of the gradient by scanning the tubes

through an UV analyzer (locally constructed) operating at a wavelength of 316 nm. The sedimentation distance at a particular EtBr concentration is divided by the distance measured at 2 μg/ml EtBr and expressed as relative sedimentation distance (rS).

Measurement of the Repair of UV-Induced DNA Pyrimidine Dimers

Cells were exposed in 30 mm plastic dishes through the 2 mm PBS supernatant with 20 J/m^2 UV light of wavelength 254 nm. This dose produces approximately 40 thymine dimers per Mbp DNA [18,19], which in repair-proficient cells are recognized by an endonuclease that cuts the strand at or near the dimer. This strand incision is the first step of excision repair and its defect in *Xeroderma pigmentosum* A, C, and D cells can clearly be observed using nucleoid sedimentation [19].

Applying the sedimentation procedure as described above (EtBr concentration constant 2 μg/ml) at various incubation times after irradiation measures the loss of supercoiled tension caused by the strand breaks.

RESULTS

EtBr-Induced Changes in DNA Supercoiling

The dependence of the relative sedimentation coefficients rS on increasing EtBr intercalation is shown for nucleoids from healthy donors (Fig. 1a) and from cancer patients (Fig. 1b). The related median values, as given in Figure 2, demonstrate that nucleoids derived from healthy donors exhibit a biphasic response. Up to 4 μg/ml EtBr there is a decrease or rS in most samples, followed by an increase at higher EtBr concentrations. This behavior reflects the loss of negative supercoiling and the buildup of positive supercoiling as depicted in the diagram (Fig. 3). In contrast to this, most of the nucleoids derived from patients are unable to develop positive supercoiling even at 8 μg/ml EtBr.

To classify each donor in respect to this supercoil reversal, the quotient

$$\frac{rS(8\ \mu g/ml)}{rS(4\ \mu g/ml)}$$

is calculated, which becomes >1 for curves exhibiting a minimum at 4 μg/ml, but is <1 for curves with a continuous decrease of rS. Using these criteria, 4 of 17 healthy donors (23%) are identified, which show this reduced ability to reverse supercoiling (S−). Among the 20 cancer patients, 17 donors (85%) exhibited this characteristic.

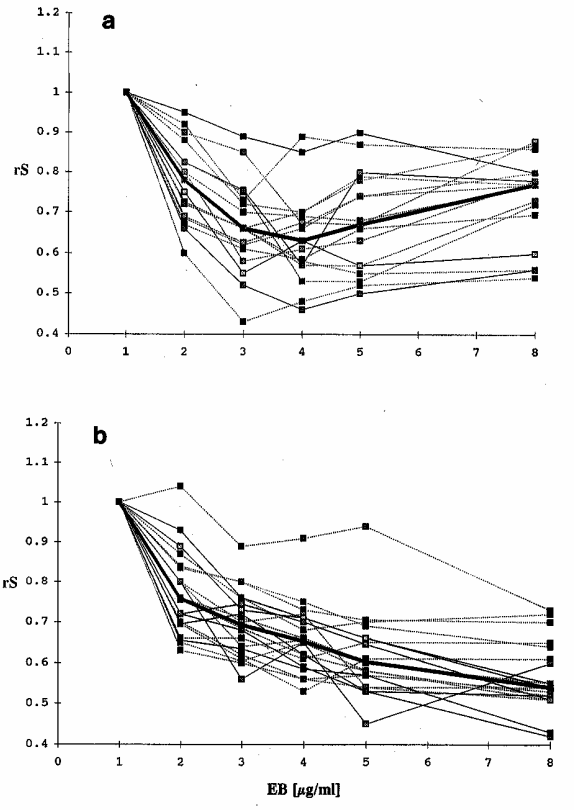

Fig. 1. Change of supercoiling after EtBr intercalation. Dependence of the relative sedimentation coefficient rS on the EtBr (EB here) concentration. Nucleoids from lymphocytes derived from healthy donors **(a)** and from cancer patients **(b)** were prepared as described in Materials and Methods. Their sedimentation distance after ultracentrifugation in different EtBr concentrations (μg/ml) was measured and divided by the sedimentation distance at 2 μg/ml EtBr (quotient called rS). The bold lines represent the median values.

Changes in DNA Supercoiling After UV Irradiation

The change of rS after UV irradiation relative to unirradiated lymphocytes is shown in Figure 4a for the healthy donors and in Figure 4b for the cancer patients. The loss of supercoiling density as reflected by decreasing rS within the first 30 min postirradiation results from enzymatic DNA strand incision at UV-induced base dimers. Comparison of the median values of both donor groups (Fig. 5) reveals that the majority of patient-derived lymphocytes do not undergo this repair-related mechanism with the same efficiency as observed for lymphocytes from healthy donors. The subsequent recov-

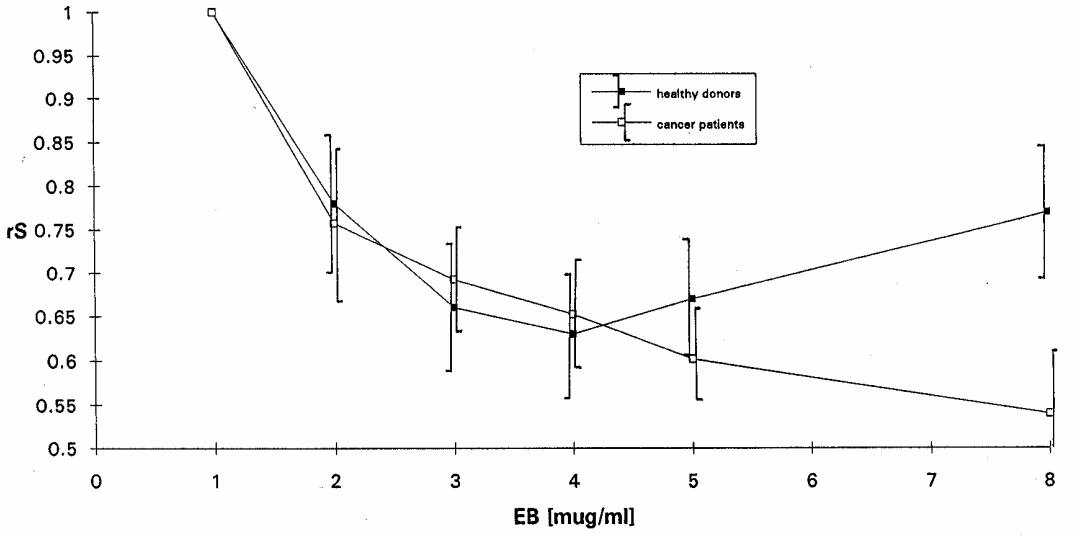

Fig. 2. Median values of the supercoiling response. The median values ⟨rS⟩ and standard deviation (SD) for the measured rS values at different EtBr (EB here) concentrations were calculated for both donor groups.

ery of rS at longer repair times, detectable in most of the lymphocytes from healthy donors, may be attributed to resynthesis and ligation of repaired DNA patches. To classify each individual donor with regard to incision kinetics, the slope of the repair curve within the first 30 min of each sample is related to the 95% confidence range of slopes in the control group. Samples exceeding a critical value of

$$rS^*_{(30\ min)} = \langle rS\rangle_{(30\ min,\ healthy\ donors)} + SD_{(30\ min,\ healthy\ donors)}$$

are considered atypical in this first essential step of UV repair (⟨rS⟩ = median value and SD = standard deviation). Under these conditions, lymphocytes from 6 of the 17 healthy donors (35%) and 8 of the 20 cancer patients (40%) are classified as exhibiting a reduction in their UV repair efficiency (R−).

Simultaneous Occurrence of Reduced Supercoiling Reversal and Reduced UV Repair

The criteria set for supercoiling stability and UV repairability allow us to look for a possible relation between both characteristics. Table 1 gives the percentages of simultaneous occurrence (S− ∩ R−) relative to the number of all group members as well as the conditioned frequency of S− in case R− (S−|R−) and vice versa (R−|S−). Obviously, a simultaneous occurrence of S− and R− is abundant in cancer patients (34%), compared to only 18% within the healthy donor group. A more detailed analysis of this finding is considered in the discussion, together with a possible explanation.

DISCUSSION

We investigated supercoiling reversal and incision kinetics for UV-damaged DNA in lymphocytes derived from 20 healthy persons and 17 cancer patients (not previously treated). The method of nucleoid sedimentation is a sensitive assay to study unlabeled, resting lymphocytes.

The ability of nucleoids to change from negative to positive supercoiling under the influence of increasing EtBr concentrations can be taken as a measure of the stability of supercoiled DNA structures. Lymphocytes, which do not exhibit this supercoiling reversal between 2 μg/ml and 8 μg/ml EtBr, are classified as suffering from impaired supercoiling stability (S−). On the other hand, the measurement of supercoiling density during UV repair allows us to identify lymphocytes with a reduced ability to recognize UV-induced DNA damages (R−).

We found significant greater impaired supercoiling density in the patient group (85%) compared to the healthy group (23%). The hypothesis that impaired supercoiling may be responsible for a reduced immune response of lymphocytes may be of interest, indicating either a causal relationship between reduced immune status and tumorigenesis or a reduced immune status as a result of the disease

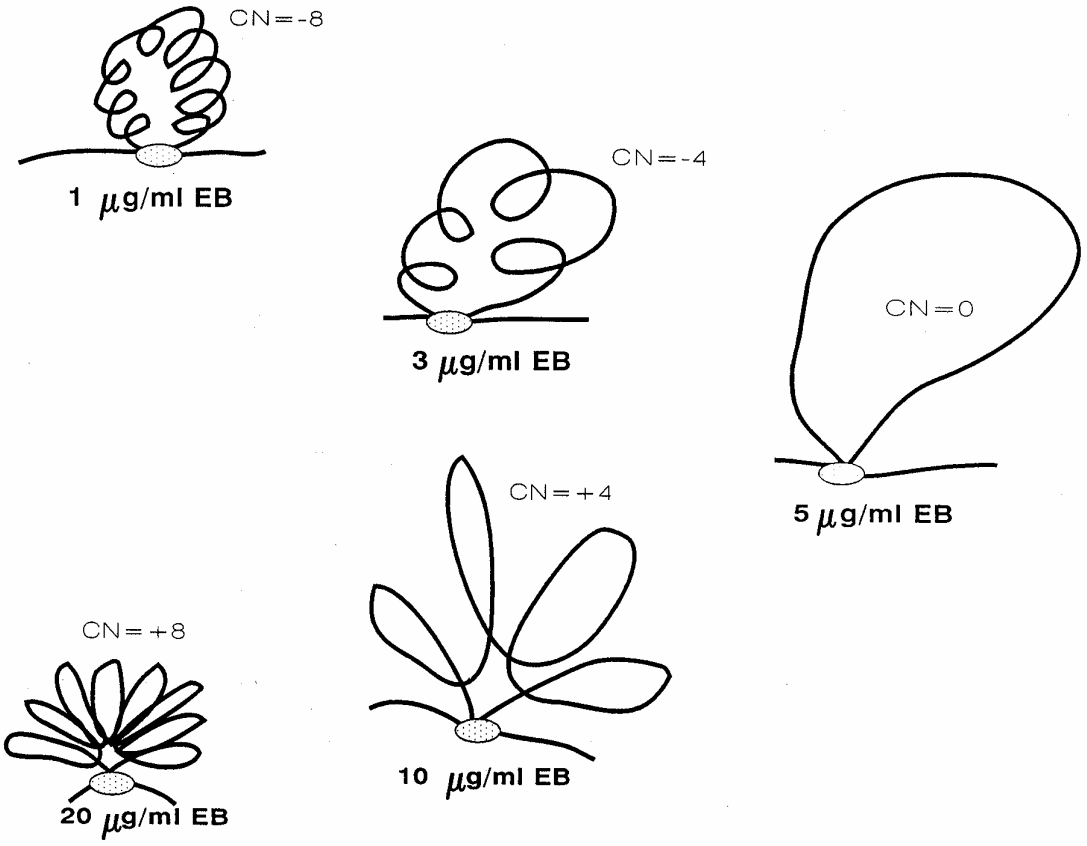

Fig. 3. Mechanism of alteration of supercoiling density by EtBr intercalation. Principle of supercoil reversal in DNA nucleoids by an intercalating dye. The primary effect of the intercalating dye EtBr lies in the alteration of the Watson-Crick DNA helix, resulting in a reduction of its helicity. Since the loop remains attached during this process, the loss of primary helicity is compensated by an increase of positive supercoiling of the DNA molecule [expressed as so-called crossing numbers (CN)]. Thus, with increasing EtBr concentration (μg/ml EB) the native negative supercoiling is lost and at higher concentrations converted into positive supercoiling **(top left to bottom left).** The CN are arbitrarily chosen values to explain the diagram.

[14]. In an earlier study, Harris et al. [20] found that lymphocytes from autoimmune disease patients exhibit a reduced chromatin density compared to healthy controls. Their conclusion, that unrepaired DNA damage might be responsible for this result, seems inconsistent with knowledge about DNA matrix binding and its possible disturbance. However, since the chromatin density might be closely related to the supercoiling stability as studied in the present paper, one has to conclude that its reduction may cause a general disorder of immune response, rather than its overall loss. Pienta et al. [7] concluded that the regulatory disorders in cancer cells which cause a heterogeneous response to various treatments may be causally related to a disturbance in their chromatin structure.

Eight of 20 patients (40%) exhibited a reduced repair capacity. This is slightly but not significantly more than that among healthy donors (35%) and less than the frequency found by Kovacs and Langemann [21]. Measuring repair of UV damage in normal and patient lymphocytes by unscheduled DNA synthesis (UDS), they observed in almost all patients a diminished repair rate for the first 2 hr following irradiation. Nevertheless, since UDS detects a later endpoint within the excision repair process than our assay does, there is no inconsistency between their and our findings. The reduced UV repairability may have consequences for the restoration of the lymphocyte pool after its reduction due to acute radiation effects. Cole et al. [3] demonstrated that the radiosensitivity of stimulated lym-

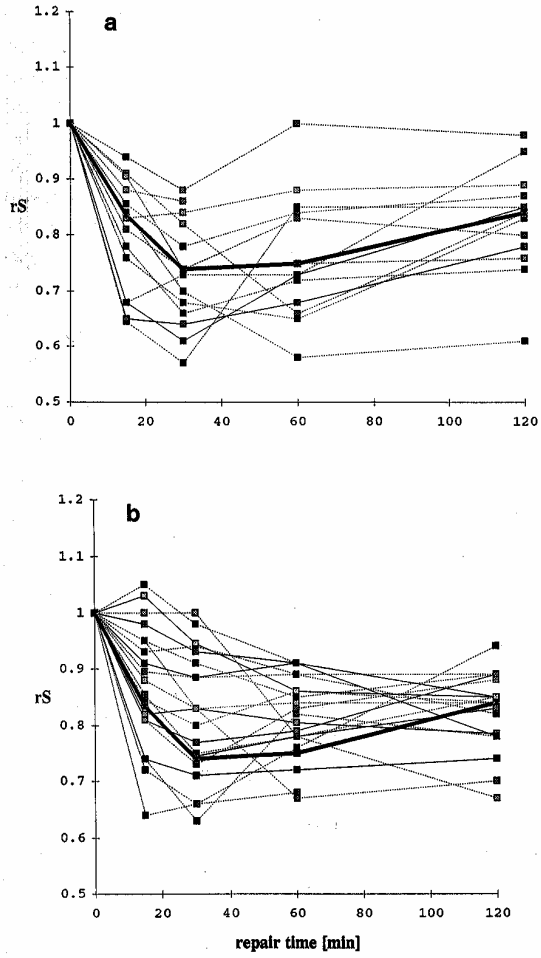

Fig. 4. Change of supercoiling during UV repair. Dependence of the relative sedimentation coefficient rS on the repair incubation time after UV irradiation in nucleoids from lymphocytes derived from healthy donors **(a)** and from cancer patients **(b)**. After UV irradiation, lymphocytes were incubated for different periods at 37°C, nucleoids were prepared as described in Materials and Methods, and ultracentrifugation was carried out at 2 μg/ml EtBr. Resulting sedimentation distances after different repair times were divided by the sedimentation distance measured immediately after UV (quotient called rS). To emphasize the difference between the two groups, the bold lines in a and b give the median values calculated from the healthy donor group.

phocytes correlates well with the sensitivity of their lymphoblastoid precursors. Unfortunately, we do not know whether there is a correlation between UV and X or γ-sensitivity of the investigated cells. Therefore, further studies must be performed to compare these experimental findings with investigations on the acute reaction following RT, focusing on leukopenia and related phenomena. Since most of the cytostatic drugs applied in chemotherapy induce UV-type DNA lesions, the recovery of the immune system from such drug-induced damage may also need the excision repair system.

Studying lymphocytes from healthy donors aged between 20 and 90 years, Hartwig and Körner [14] found a significant increase of negative supercoiling and a reduction of the incision kinetics after UV irradiation with advancing donor age. However, within the rather narrow age distribution of our donors (50–73 years, with 85% between 56 and 70 years) we did not find a significant age dependence of either the supercoiling characteristics or the UV repair kinetics (data not shown). Furthermore, the age distributions of the control group and of the patient group were comparable. Thus the higher portion of patients with impaired supercoiling cannot be attributed to an age-related phenomenon.

The simultaneous occurrence of S− and R− in the healthy donor group allows some conclusions on a correlation between both factors, since the observed frequencies ($S- \cap R- = 18\%$, $R-|S- = 75\%$, $S-|R- = 50\%$) are significantly higher ($P > 0.95$) than the related values expected for double random distributions of both characteristics ($S- \cap R- = 8\%$, $R-|S- = 36\%$, $S-|R- = 24\%$). Studying lymphocytes from autoimmune disease patients, Harris et al. [20] concluded that chromatin structure in unirradiated cells may be altered due to the reduced repair of endogenously induced DNA damage. Our finding that altered supercoiling stability in 75% of all cases is connected with reduced repair, whereas reduced repair is connected with altered supercoiling in only 50% makes it more likely that a repair deficiency is caused by altered supercoiling than vice versa. However, as with any correlation it cannot be decided if this simultaneous occurrence reflects a causal relationship between altered chromatin structure and reduced DNA repair [10] or if both abnormalities are only epigenetic. Other factors must be involved which are responsible for the remaining three cases of reduced repair which we found not to be related to altered supercoiling. It is well known that a genetic defect for a single enzyme can cause repair deficiencies.

Interestingly, the simultaneous occurrence of both abnormal DNA supercoiling and reduced repair capacity is relatively common in the patient group: 34% of patient-derived lymphocytes with reduced repair capacity also exhibit reduced supercoiling stability (Table 1). Although this percentage is significantly higher than among healthy donors, the actual number is not higher than the expect value for a random distribution of both parameters.

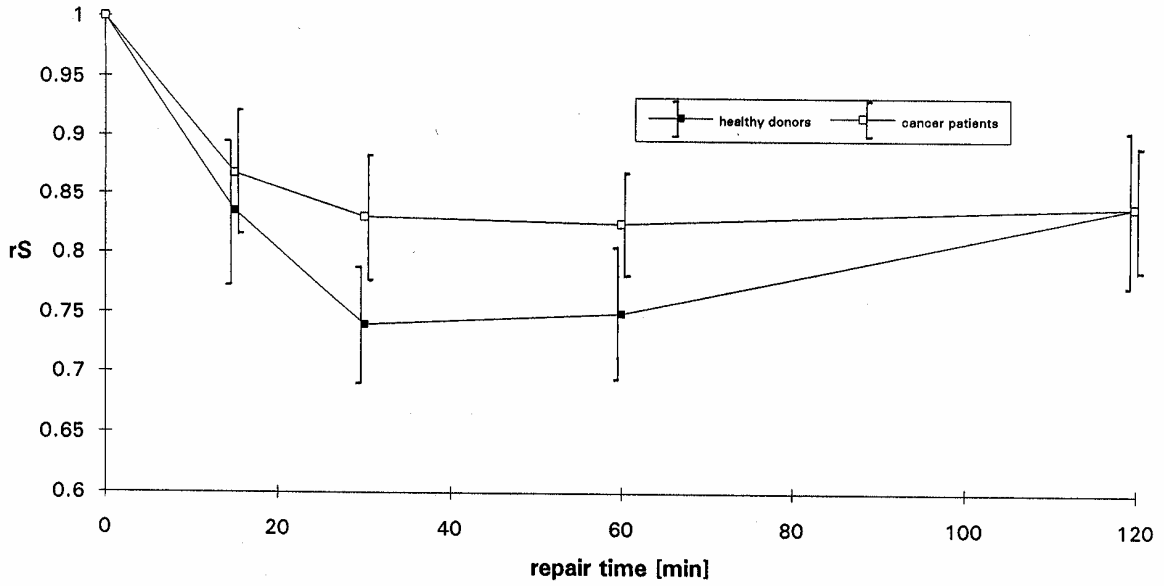

Fig. 5. Median values of supercoiling changes during UV repair. The median values ⟨rS⟩ and standard deviation (SD) for the measured rS values at different repair times after UV irradiation were calculated for both donor groups.

Table 1. Frequencies for Separate Occurrence of Reduced Repair Capacity (R−) and Altered Supercoiling (S−) in Lymphocytes of Both Donor Groups and for Simultaneous Occurrence of the Two Abnormalities†

	Observed frequency f (%)	Calculated frequency Φ assuming random distribution of R− and S− (%)	Critical value for f (T-test for $P > 0.95$) (%)
Healthy donors			
S−	23		
R−	36		
(R− ∩ S−)	18	8	12
(R−|S−)	75	35	45
(S−|R−)	50	24	29
Cancer patients			
S−	85		
R−	40		
(R− ∩ S−)	34	35	44
(R−|S−)	41	40	50
(S−|R−)	88	85	98*

†(R− ∩ S−) expresses the frequency of simultaneous occurrence of both abnormalities relative to the overall number of donors in each group. (R−|S−) expresses the frequency of reduced repair (R−) only in donors with an altered supercoiling (S−) and (S−|R−) vice versa. Critical values are calculated using the T-test. They give the lower limit for f to assume correlation between R− and S− (0.95 confidence).
*$P > 0.80$.

The same holds true for the conditioned frequencies (S−|R− = 88%) and (R−|S− = 41%), neither of them exceeding the theoretical values for a double random distribution (85% and 40%, respectively). This means that for the patient group we cannot draw any conclusion on a causal relationship between S− and R−. Nevertheless, for any practical purpose one may assume that donors showing both a reduced repair capacity and an altered chromatin structure carry an additional risk factor for the radiation response of their lymphocyte pool.

Knowledge of an abnormal immune status or of a reduced DNA repair rate may have consequences for treatment planning, especially since

both RT and chemotherapy result in an additional suppression of the immune system. The state of the immune system is recognized today as a crucial factor for any tumor therapy.

Both parameters studied here are rather easy to derive and may represent a useful additional prognostic marker prior to treatment. To confirm the value of this approach, we are currently carrying out a follow-up study on the long-term therapeutic success of the patients whose lymphocytes were included in this study.

CONCLUSIONS

Lymphocytes obtained from previously untreated cancer patients show a significantly higher frequency of excision repair defects and alterations in their supercoiling density (chromatin structure) than do lymphocytes from healthy donors. There is a high proportion of cancer patients exhibiting the simultaneous occurrence of both characteristics. Both symptoms are assumed to play a major role in the radiation response of lymphocytes, either due to the maintenance of functional integrity or for their contribution to recovery after radiation damage. The heterogeneity within both groups with respect to DNA repair and chromatin structure may be especially important for the patient group, since any therapy induces an additional stress for the immune system, the functional integrity of which plays a crucial role in overall therapeutic success. A prognostic assay like the one presented in this study may be included in the treatment planning in an attempt to minimize acute reactions of sensitive patients.

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