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BIOLOGY CONTRIBUTION

INCREASED BUT ERROR-PRONE NONHOMOLOGOUS END JOINING IN IMMORTALIZED LYMPHOBLASTOID CELL EXTRACTS FROM ADULT CANCER PATIENTS WITH LATE RADIONECROSIS

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Purpose: **To study nonhomologous end joining in extracts of two lymphoblastoid cell lines derived from patients with late radionecrosis after radiotherapy. Both cell lines were previously shown to exhibit impaired rejoining of DNA double-strand breaks in a pulse-field gel electrophoresis assay.**

Methods and Materials: **We used a cell-free system and quantitative real-time polymerase chain reaction, as well as sequencing analysis of end joining products.**

Results: **Paradoxically, extracts of the two cell lines display increased rates of *in vitro* end joining of noncohesive termini compared with normal cell extracts. This increase was seen in the absence of added deoxyribonucleoside triphosphates and was sensitive to inhibition by wortmannin. Sequencing of the joined products revealed that, despite increased rates of end joining, the process was error prone with a greater frequency of deletions compared with that observed in normal controls.**

Conclusion: **These findings are consistent with the suggestion that a promiscuous, deletion-prone abnormality of nonhomologous end joining might underpin the predisposition of certain radiotherapy patients to late radionecrosis. We hypothesize that some individuals might harbor subclinical defects in nonhomologous end joining that clinically manifest on challenge with high-dose radiation. Because both quantitative and qualitative aspects of end joining have demonstrably been influenced, we recommend that the study of patient samples should involve a combination of quantitative methods (*e.g.,* quantitative real-time polymerase chain reaction), sequencing analysis, and a comparison of multiple join types.** © **2008 Elsevier Inc.**

Radiation injury, Nonhomologous end joining, Error-prone repair.

INTRODUCTION

The radiation dose is limited by the normal tissue tolerance; at the radiation dose schedules tolerated by most individuals, some patients develop late radionecrosis. Most of these present with no preradiation phenotype. Our knowledge of the factors accounting for individual differences in response to radiotherapy is rudimentary (1).

We have established five lymphoblastoid cell lines derived from previously healthy individuals who presented with cancer at <50 years of age and who subsequently developed late radionecrosis after standard radiotherapy. Two of these cell lines, LB0004 (2) and LB0005, demonstrated increased *in vitro* radiation sensitivity. In an annexin V/flow cytometric assay, LB0005 cells were shown to be more radiosensitive than control cells (data not shown). Postirradiation DNA repair, as measured by pulsed-field gel electrophoresis, was also defective. In particular, the ability to carry out double-strand break (DSB) repair by the fast kinetic pathway (3) was significantly reduced in both LB0004 (2) and LB0005. After 30 Gy radiation, in LB004, just 5% of the DNA fluorescence had moved into the gel 30 minutes after irradiation compared with about 40% for control cell lines (2). In LB0005, the rate of DNA migration into the well was also reduced, similar to LB0004 (unpublished observation).

The major pathway of DNA DSB repair in mammalian cells, responsible for mediating fast kinetics, is nonhomologous end

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joining (NHEJ) (4). We, therefore, hypothesized that defects in NHEJ, observed in cell-free extracts from LB0004 and LB0005, contribute to the abnormal kinetics of DNA DSB repair, as measured by pulsed-field gel electrophoresis, and that these NHEJ defects might also underlie the predisposition to late radionecrosis manifested by the patients.

Recently, Budman and Chu (5) described an *in vitro* assay for measuring NHEJ efficiency in cell-free extracts. Their method uses real-time quantitative PCR (qPCR) to measure the efficiency of end joining, instead of the previously used gel-based visualization methods (6). We reasoned that the sensitivity of the qPCR assay (which can quantify the end-joined DNA for nine orders of magnitude) might be useful for detecting subtle NHEJ defects and applied the strategy to measure the products of end joining by cell-free extracts derived from LB0004 and LB0005.

Processing of DNA ends is another key step during NHEJ, because agents such as ionizing radiation create ends that cannot be directly ligated. As such, the assay uses a variety of DNA ends: ends with 5′-overhangs (*e.g.,* BamHI) and 3′-overhangs (*e.g.,* KpnI), as well as blunt ends (*e.g.,* StuI and EcoRV). The pairs of BamHI ends and KpnI ends are described as cohesive, because the ends can be directly ligated and the overhanging sequences can engage in appropriate Watson-Crick base pairing. A pair of blunt ends (*e.g.,* StuI and EcoRV) can be directly ligated. All three pairings can be described as compatible, because no processing is needed before ligation can occur.

In contrast, the pairing of a 5′-overhang end with a 3′-overhang end cannot be ligated directly and requires processing of the ends before ligation can proceed. This hurdle also exists when either a 5′-overhang end or 3′-overhang end is paired with a blunt end. Such pairings can be described as noncompatible (Fig. 1).

Paradoxically, the efficiency of end joining of noncompatible ends was significantly increased in LB0004 and LB0005, in both the absence and the presence of added deoxyribonucleoside triphosphates (dNTPs). This contrasted sharply with observations of cell extracts from the DNA-PKcs–deficient cell line M059J (7), which showed significantly reduced end joining efficiency for noncompatible ends.

Sequencing of the join products of noncompatible and blunt ends revealed that LB0004 and LB0005 extracts processed the ends in a manner that is deletion prone compared with the normal control cell line. Our data indicate that increased, but error-prone, end joining in cell-free extracts from LB0004 and LB0005 might underlie a predisposition to late radionecrosis in these patients.

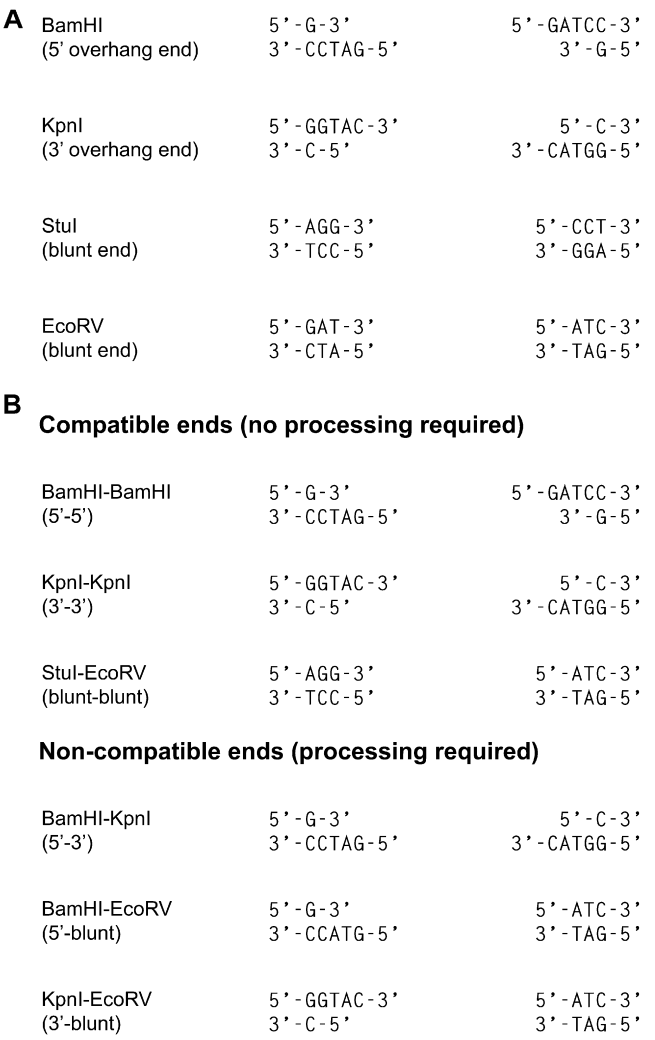

Fig. 1. Diagrammatic representation of various DNA substrate ends and end pairings. (A) DNA substrate ends. Digestion by BamHI of its restriction site yielded DNA end with 5′-overhang. KpnI yielded 3′-overhang. Both StuI and EcoRV yielded blunt ends. (B) Classification of end pairings as compatible and noncompatible. Pair of BamHI ends was compatible, because overhangs can form canonical Watson-Crick base pairing, and ends were directly ligate-able. Same applies to pair of KpnI ends. Direct ligation was also possible in pairing of StuI end and EcoRV end. In case of end pairings involving BamHI and KpnI, BamHI and EcoRV, and KpnI and EcoRV, end were not directly ligate-able and were thus noncompatible. Ends must, therefore, be processed before ligation can proceed.

METHODS AND MATERIALS

Cell-free extracts and end joining reaction

SNC3 is a lymphoblastoid cell line with normal *in vitro* radiation sensitivity and DNA DSB repair kinetics (data not shown). It was derived by Epstein-Barr virus immortalization from a healthy volunteer. LB0004 (2) and LB0005 were derived from 2 cancer patients who developed late radionecrosis. As a negative control cell line, we used M059J, a radiosensitive glioma cell line with no DNA-PKcs expression (7) and reduced NHEJ *in vitro* (8). Cell-free extracts were prepared according to the micro-scale method previously described (9), using 1–2 $\times$ 10^8 cells for each extraction. The protein concentrations were 10–20 mg/mL. End joining reactions were performed according to Diggle *et al.* (9), with the reaction incubated at 37°C for 2 h. The joined DNA was purified using a Qiagen MinElute column. To assess both the proportion of DNA recoverable from the column and the reproducibility of measurements between columns, standardized amounts of control DNA were loaded and eluted, and a calibration curve was plotted (data not shown). As indicated, before substrate addition, the cell extracts were pretreated with wortmannin (100 μM final concentration, Sigma Chemical, St. Louis, MO) for 30 min on ice. Wortmannin was prepared in 10% dimethyl