Elevated Levels of Rad51 Recombination Protein in Tumor Cells

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ABSTRACT

Rad51 is the key enzyme for homologous recombination, an evolutionarily conserved mechanism for the repair of DNA damage and the generation of genetic diversity. Given the observation that many tumors become resistant to radiation therapy and DNA-damaging chemotherapeutics and also that tumor cell populations can acquire a high number of genetic alterations and then expand clonally, dysfunction of the mammalian Rad51 recombinase could play a major role in the multistep process of tumorigenesis. The data we present provide further strong support for this hypothesis. Using anti-Rad51 immunofluorescence staining, widely different tumor cell lines displayed increased numbers of nuclei with focally concentrated Rad51 protein compared with nonmalignant control cell lines. These nuclear foci are thought to represent a repairosome-type assembly of Rad51 and other proteins required for recombinational DNA repair. By Western blot analyses, the net amount of Rad51 protein was increased 2–3-fold in all tested tumor cell lines. Inhibition of de novo protein synthesis by cycloheximide treatment showed a similar half-life of Rad51 protein in normal and tumor cells. Fluorescence in situ hybridization experiments did not detect Rad51 gene amplifications in tumors. Because Northern blot analysis demonstrated highly elevated Rad51 mRNA levels, we conclude that the increases in Rad51 protein and nuclear foci formation in tumor cells are the result of transcriptional up-regulation.

INTRODUCTION

Rad51, a structural and functional eukaryotic homologue of Escherichia coli RecA recombinase, is the key enzyme for DNA DSB repair by homologous recombination. Similar to RecA, both yeast and mammalian Rad51 proteins form nucleoprotein filaments on ssDNA, mediating homologous pairing and strand exchange reactions between ssDNA and homologous double-stranded DNA (1–4). Mammalian Rad51 appears to function as part of a larger recombination complex that includes Rad52 (5) and Rad54 (6), which belong to the same epistasis group as Rad51, and replication protein A (7, 8).

Previously, it was thought that, in contrast to yeast, mammalian cells repaired DSBs primarily through nonhomologous end-joining of the broken ends (9). However, accumulating experimental evidence suggests that the homologous recombination pathway is equally important (10, 11). In particular, in highly replicating mammalian cell types, homologous recombination might play a predominant role for DSB repair (12). Rad51-deficient chicken lymphocytes in which a human Rad51 transgene was inactivated showed increases in chromosome breaks and cell death (13), whereas Rad51-overexpressing mammalian cells showed an increased resistance to ionizing radiation (14) and DNA damage-induced apoptosis.4 Whereas Escherichia coli RecA and yeast Rad51 mutants are viable, disruption of both Rad51 alleles in the mouse led to early embryo lethality, and knockout cells displayed genomic instability, progressive chromosome loss, and cell death (15, 16). One possible explanation is gain of function of Rad51 protein in mammalian cell proliferation and/or genome metabolism.

Observations that mammalian Rad51 protein associates with the tumor suppressors Atm (17), p53 (18), Brc1 (19), and Brc2a (20); the c-Ab1 oncogene product (21); and the Blm helicase (22) suggest its probable role in tumor development. Aberrant overexpression of Rad51 protein could confer several advantages to tumor cells. First, the DNA repair function of Rad51 may protect cells from DNA damage and apoptosis. Secondly, overstimulation of homologous recombination and chromatid exchange mechanisms by Rad51 protein (23, 24) may contribute to genomic instability and genetic diversity of tumor cells. In this study, we demonstrate elevated levels of Rad51 protein in a variety of tumor cell lines.

MATERIALS AND METHODS

Cell Culture. Primary and SV40-transformed human fibroblasts, as well as most tumor cell lines used in this study (Table 1), were grown as monolayer cultures in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, antibiotics, and, when necessary, vitamins and various growth factors. EBV-transformed lymphoblasts and blood tumor cell lines were grown in suspension in supplemented RPMI 1640 (Life Technologies, Inc.). To induce apoptosis, etoposide (4 μg/ml) was added to the culture medium for 24 h. Etoposide interferes with topoisomerase II, an enzyme that binds covalently to double-stranded DNA, cleaves both strands, and resells the cleaved complex. Etoposide hampers with this breakage and rejoining cycle by trapping the enzyme in the cleaved complex. Irreparable DSBs are then formed, and cell death follows (25). To block de novo protein synthesis, cycloheximide was added to the cultures for 8 and 24 h at a final concentration of 10 μg/ml (26).

Immunoblot Analysis. To compare the net amounts of Rad51 protein between various normal and tumor cell lines, cells were harvested during the logarithmic growth phase. From monolayer cultures, both cells attached to the culture flask and apoptotic cells in suspension were collected. Harvested cells were lysed in PBS [PBS = 136 mm NaCl, 2 mm KCl, 10.6 mm Na2HPO4, and 1.5 mm KH2PO4 (pH 7.3)] containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor (Boehringer Mannheim) and disrupted with Qiagen shredder columns. Proteins were precipitated with acetone and resuspended in 25 μl of sample buffer consisting of 50 mm Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.004% bromphenol blue.

Protein extracts were resolved by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The resulting filters were blocked overnight with 5% nonfat dried milk, incubated with the appropriately diluted rabbit anti-Rad51 antisera (27) for 24 h, incubated with horseradish peroxidase-conjugated antirabbit IgG (Dianova), and washed. Antibody binding was visualized by chemiluminescence (ECL RPN 2209; Amersham). Although equal amounts of total cellular protein were loaded per lane, all filters were reincubated with anti-β-actin antibody (Sigma Chemical Co.) to compare the protein levels in different cell substrates. The intensity of the Rad51 signals was equilibrated to the intensity of the β-actin signals using PCbas2.0 software.

Northern Blot Analysis. Total RNAs were isolated from tumor and control cell lines with the RNeasy midi kit (Qiagen) and subsequently used for mRNA isolation with Oligotex beads (Qiagen). Three 18 U.S.C. Section 1734 solely to indicate this fact.

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6 The abbreviations used are: DSB, double-strand break; ssDNA, single-stranded DNA; FISH, fluorescence in situ hybridization; YAC, yeast artificial chromosome.


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**Immunofluorescence Staining.** Harvested cells were washed and resuspended in PBS. Aliquots (10⁶ cells in 0.5 ml of PBS) were centrifuged onto clean glass slides using a Shandon Cytospin. Immediately after cytospin- gation, the preparations were fixed in absolute methanol for 30 min at −20°C and then rinsed in ice-cold acetone for a few seconds. After three washes with PBS, the slides were incubated with blocking solution (3% BSA, 0.1% Tween 20, and 4× SSC) in a Coplin jar for 30 min and then incubated with rabbit anti-Rad51 antiserum (diluted 1:100 with PBS) in a humidified incubator for 30 min. The slides were then washed in PBS after three times for 10 min each and incubated for 30 min with FITC-conjugated antirabbit IgG appropriately diluted with PBS. After three additional washes with PBS, the preparations were counterstained with 1 μg/ml 4’,6-diamidino-2-phenylindole in 2× SSC for 1 min. The slides were mounted in 90% glycerol, 0.1 M Tris-HCl (pH 7.0) at 60°C and washed once for 5 min in 0.1× SSC, 3% BSA, and 0.1% Tween 20 at 37°C for 30 min. Probes were detected with FITC-avidin (Vector Laboratories) and Cy3-conjugated antidigoxin antibodies (Dianova).

**RESULTS**

**Increased Numbers of Nuclear Rad51 Foci in Tumor Cells.** Previously, we have shown that in normal human cell cultures, Rad51 protein is detected in multiple discrete foci in the nucleoplasm of a small number of cells. After DNA damage, the percentage of cells with focally concentrated Rad51 protein increased in a time- and dose-dependent manner (27, 30). Circumstantial evidence suggests that Rad51 recruitment takes place from endogenous Rad protein dispersed throughout the nucleus to damaged chromatin in S-G2 phase (31–33). In contrast, stably transfected cells that constitutively overexpress Rad51 protein form nuclear foci in the absence of DNA damage, most likely by self-interacting Rad51 molecules (34, 35). The overexpressed Rad51 protein confers resistance to DNA damage-induced apoptosis. In addition, Rad51 foci were observed in meiotic prophase cells (36, 37), which undergo high levels of genetic recombination, and in splenic B cells (38), which are activated for antibody class switch recombination. Collectively, these observations suggest that Rad51 foci are associated with DNA repair and/or stimulation of homologous recombination.

Given the hypothesis that increased recombinational DNA repair is advantageous for tumor cells, formation of repairosome-type Rad51 foci might also occur during tumorigenesis. Here we have determined the number of Rad51 foci in widely different tumor cell lines, including leukemias, carcinomas, melanomas, and glioblastoma, by anti-Rad51 immunofluorescence. Compared with nonmalignant cell sub-
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Insights into the mechanisms underlying the development of radiation/chemotherapy resistance and the increased chromosome mutation rate could help to improve diagnosis and treatment of tumors. Because the Rad51 recombinase is critical to DSB repair and the generation of genetic diversity in higher eukaryotes, it might also contribute to the development of cancer. The potential functional

strates (lymphoblasts and primary and transformed fibroblasts), most tumor cell lines exhibited an increased percentage of nuclei with Rad51 foci (Table 1). Type I nuclei showed many (>10) discrete foci scattered throughout the entire nuclear volume (Fig. 1A), whereas type II nuclei contained clusters of brightly fluorescing foci that were highly enriched with Rad51 protein (Fig. 1B). In particular, the type II foci were very rare in nonmalignant cell substrates.

Elevated Rad51 Protein Levels in Tumor Cells. The observed accumulation of Rad51 protein in nuclear foci could be due to a net increase in Rad51 protein in tumor cells or to a relocalization of dispersed Rad51 protein to nuclear foci. To determine the Rad51 protein levels in tumor versus normal cells, we have performed quantitative Western blot experiments. In primary fibroblasts, anti-Rad51 antibodies detected one predominant band at 39 kDa, which corresponds to the full-length Rad51, and another much weaker band at approximately 37 kDa (Fig. 2). After induction of DNA damage by etoposide treatment (4 μg/ml for 24 h), the intensity of the 39-kDa band and, consequently, the net amount of total Rad51 protein drastically decreased. This demonstrates that formation of DNA damage-induced Rad51 foci is due to redistribution of the protein (27, 33), but not to de novo protein synthesis. In contrast, the intensity of the faster-migrating 37-kDa band remained unchanged or slightly increased after etoposide treatment of primary cells. It has been speculated that the lower band is a product of proteolytic cleavage during apoptosis (39). Cleavage of Rad51 by caspase 3 after DNA damage results in loss of its recombinase activity (40).

By Western blotting, there was a 2–7-fold increase in full-length Rad51 protein in all tumor cell lines compared with primary (PF1 and PF2) cells (Fig. 3). Because of the very prominent 39-kDa band, the lower 37-kDa band was hardly visible. The observation that the amount of the proteolytic Rad51 cleavage product did not increase argues against the notion that the elevated Rad51 protein levels are the result of high levels of DNA damage in tumor cells. After Rad51 protein staining, the same filters were reincubated with anti-β-actin antibody. The β-actin signals were used to equilibrate slightly different amounts of cell extract loaded per lane. To determine the half-lives of Rad51 protein in primary (PF1) fibroblasts and representative tumor (SkBr3 and Mel2a) cells, cultures were treated with cycloheximide for 8 and 24 h (Fig. 4). In the absence of de novo protein synthesis, the Rad51 protein level decreased approximately 30% in 1 day. There was no detectable difference in protein stability between primary and tumor cells.

Fig. 1. Immunofluorescence staining of Rad51 protein in Mel2a (A; type I foci) and EM2 (B; type II foci) tumor cells, representing the two different Rad51 foci types.
association of mammalian Rad51 protein with tumor suppressors, including Atm (17), p53 (18), Brca1 (19), and Brca2 (20), has led to the proposal that Rad51 also may be a tumor suppressor. Interestingly, in addition to their colocalization with Rad51 to nuclear foci, the breast cancer predisposition gene products Brca1 and Brca2 may themselves participate in the repair of DNA damage. Cells with defective Brca1 or Brca2 alleles displayed radiation hypersensitivity and an increased number of chromosome breaks (20, 41–43). However, the fact that the Rad51 gene is not targeted by mutations in tumors (44, 45) does not support a role for Rad51 as a tumor suppressor. Interaction of the c-Abl oncogene product with Rad51 may be required for the correct posttranslational modification of Rad51 and the assembly of DNA repair protein complexes (17, 21).

Our results demonstrate increased levels of Rad51 protein and increased numbers of cells with nuclear Rad51 foci in a wide variety of tumor cell lines. Two conceptually related studies (46, 47) found that wild-type Rad51 protein is up-regulated in pancreatic adenocar-

Table 2 Number of Rad51 loci/haploid genome
At least 20 metaphases were analyzed per cell line.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chromosomes/metaphase</th>
<th>Rad51-YAC signals/metaphase</th>
<th>Rad51 genes/haploid genome</th>
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<tbody>
<tr>
<td>K562</td>
<td>65</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Hey</td>
<td>46</td>
<td>1.5</td>
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<td>SkBr3</td>
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<td>1.5</td>
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</tr>
<tr>
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<td>2.4</td>
<td>0.9</td>
</tr>
<tr>
<td>MeWo</td>
<td>50</td>
<td>2.0</td>
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</tr>
<tr>
<td>Bro</td>
<td>55</td>
<td>1.8</td>
<td>0.8</td>
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</table>

* Number of YAC signals/23 chromosomes.

Fig. 5. FISH with a Rad51-containing YAC (red Cy3 signals) and a chromosome 15-specific DNA library (green FITC fluorescence) on normal PF1 (A) and representative LS180 (B), MeWo (C), and SkBr3 (D) tumor metaphase spreads. Chromosomes are counterstained with 4′,6-diamidino-2-phenylindole (blue).
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We have previously shown (33) that after induction of DNA damage and during meiotic prophase, nuclear Rad51 foci are formed at sites of ssDNA. Because most models of recombination and repair involve a ssDNA intermediate, DNA damage-induced and meiotic Rad51 foci are thought to represent active sites of recombinational DNA repair. Consistent with this hypothesis, Rad52 (5), Rad54 (6), replication protein A (7, 8), Xrc2 (49), and Xrc3 (50) all of which facilitate Rad51-mediated homologous recombination, are also highly enriched in these nuclear Rad51 foci types. However, in tumors, mechanisms other than induction of the DNA damage response pathway by elevated DSB levels are very likely to be at work. In normal cells, the formation of Rad51 foci after γ-irradiation or etoposide treatment is not associated with increased Rad51 protein levels (27, 33), suggesting posttranslational modification. In contrast to DNA damage-induced redistribution of Rad51 to ssDNA (32, 33), fibroblasts stably infected with the human Rad51 gene exhibited a high number of nuclei with Rad51 foci even in the absence of DNA damage.3 In fact, a 2-fold increased Rad51 protein level was already sufficient to induce foci formation by self-interaction of overexpressed Rad51 molecules. Similar to the situation in stably transfected cell lines, the constitutive overproduction of Rad51 in tumors can be expected to destroy the balance between different components of the DNA repair system. Tumor suppressor proteins such as p53 (18) and Brca2 (20), which interact with Rad51 directly, are thought to keep Rad51 in an inactive monomeric state. When Rad51 molecules are overexpressed and/or tumor suppressors become functionally inactivated in tumor cells, Rad51 may form multimeric complexes because of potentially limiting concentrations of interacting tumor suppressor proteins. Although the Rad51 protein steady-state level and Rad51 foci formation are interrelated, they may be regulated in different ways and may not exhibit a simple linear relationship. Foci formation after DNA damage occurs despite decreased amounts of full-length Rad51 protein. On the other hand, the dramatically increased Rad51 protein levels in some tumor cell lines (i.e., Hey and LS180) were not associated with increases in the number of Rad51 foci-positive cells. Defects in Rad54 (6), Xrcc2 (49), Xrcc3 (50), or other genes required for assembly and stabilization of multimeric Rad51 protein complexes may account for this defect in Rad51 foci formation.

Cycloheximide treatment for 24 h led to a comparable (relative) decrease in full-length Rad51 protein in normal and tumor cells. Thus, elevated protein levels are not likely to be due to a prolonged half-life of Rad51 in tumor cells. In contrast, the highly elevated Rad51 mRNA levels in Northern blots suggest that the Rad51 gene is transcriptionally up-regulated in tumor cells. By FISH we have excluded the possibility that Rad51 gene amplifications and gross chromosomal changes involving the Rad51-containing region 15q14–15 account for the increased Rad51 expression. Interestingly, the Rad51 mRNA levels appeared to be even more increased than the Rad51 protein levels, which is consistent with a tight regulation of Rad51 not only on the transcriptional level but also on the protein level.

The increased amounts of full-length Rad51 protein in tumor cells and possible interactions with other proteins and/or ssDNA result in nuclear Rad51 foci. Similar to the overexpressed Rad51 protein in stably infected cell lines,6 the increased amount of Rad51 protein may protect the tumor cells from undergoing apoptosis in response to DNA damage. In addition, increased Rad51 protein levels may be associated with enhanced recombination and genomic instability. The generality of Rad51 up-regulation and foci formation in widely different tumors suggests that this is not only a secondary phenomenon but is implicated in malignant transformation and/or tumor progression. Interestingly, and in contrast to constitutive overexpression of Rad51 in stably transfected cell lines and tumors, abrupt Rad51 overexpression under the control of a repressible promoter resulted in a decreased growth rate and an increased apoptotic rate (51). These cytotoxic and cytokinetic effects of acute Rad51 overexpression are thought to protect multicellular organisms against hyperrecombination and genomic instability. Evidently, tumor cells become adapted to increased Rad51 protein levels during clonal selection and tumor evolution. However, additional studies will be required to determine at which point during the multistage process of tumorigenesis Rad51 up-regulation occurs and to understand its clinical significance. Our findings have possible diagnostic and therapeutic applications. Firstly, Rad51 could serve as a diagnostic/prognostic marker to improve tumor classification. More importantly, down-regulation of Rad51 protein by Rad51 antisense oligonucleotides (52, 53) or Rad51-inhibitory drugs could be used to sensitize tumors to radiation or chemotherapy.

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REFERENCES


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