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Deregulated DNA Polymerase β Induces Chromosome Instability and Tumorigenesis[1]

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ABSTRACT

To reach the biological alterations that characterize cancer, the genome of tumor cells must acquire increased mutability resulting from a malfunction of a network of genome stability systems, *e.g.*, cell cycle arrest, DNA repair, and high accuracy of DNA synthesis during DNA replication. Numeric chromosomal imbalance, referred to as aneuploidy, is the most prevalent genetic changes recorded among many types of solid tumors. We report here that ectopic expression in cells of DNA polymerase β, an error-prone enzyme frequently over-regulated in human tumors, induces aneuploidy, an abnormal localization of the centrosome-associated γ-tubulin protein during mitosis, a deficient mitotic checkpoint, and promotes tumorigenesis in nude immunodeficient mice. Thus, we find that alteration of polymerase β expression appears to induce major genetic changes associated with a malignant phenotype.

INTRODUCTION

Tumor development proceeds via a process in which a succession of genetic alterations, each conferring growth advantage, leads to the progressive conversion of normal cells into cancer cells (1). In normal cells, DNA replication and the partitioning of chromosomes are exceptionally accurate processes. During every division cycle, each daughter cell receives full and exact genetic information, and inaccuracy of such transfer may result in the many mutations present in a variety of human malignancies. To account for the disparity between the rarity of mutations in normal cells and these elevated genetic alterations that characterize cancer, the concept of a mutator phenotype was formulated (2). According to this hypothesis, the genome of tumor cells must acquire increased mutability resulting from a malfunction of a network of genome stability systems, *e.g.*, cell cycle arrest, DNA repair, and high accuracy of DNA synthesis during DNA replication. Together, these highly conserved functional pathways act to limit cancer risk. In the course of the identification of the possible events that lead to a mutator phenotype, we identified previously a new category of genetic occurrence that can increase the genetic instability at the nucleotide level in mammalian cells (3): the overexpression of the error-prone DNA Pol β,[3] which is observed in several human malignancies. Pol β is one of the structurally simplest of the known mammalian DNA polymerases, which is believed to function primarily in the repair of damaged bases in normal somatic cells (4). It is a monomeric protein of 335 amino acids (M_r 39,000) that lacks exonuclease activities. At the transcriptional level, Pol β is overexpressed in many cancer cells (5). High levels of Pol β have also been detected at the protein level in ovarian tumors (6), as well as prostate, breast, or colon cancer tissues, compared with adjacent normal tissues (7). Furthermore, Pol β levels and activity are increased in chronic myelogenous leukemia patients.[4]

In most cases, the genetic instability of solid tumors is observed not at the nucleotide level but at the chromosomal level, with frequent gains and losses of whole chromosomes (8) concomitantly to a defect of centrosome, which play an important role in spindles assembly for chromosomal segregation (9, 10). Here, we found that overexpression of DNA Pol β could also affect chromosome stability and directly participate in cancer-associated changes.

MATERIALS AND METHODS

Cell Transfections and Immunoblotting. Pol β-overexpressing plasmid pUTpolβ was constructed after a procedure described previously (11). *Pol β* cDNA was fused in frame with the bacterial *Sh::ble* gene conferring resistance to the broad-spectral zeocin xenobiotic of the phleomycin family. The fusion did not alter Pol β expression. Selection of the transfected clones was achieved in the presence of 500 μg/ml zeocin, and the cells were then maintained in medium containing 250 μg/ml zeocin. CHO-AA8 cells were grown as monolayers at 37°C in minimum essential medium supplemented with glutamine, 8% FCS, and antibiotics. Expression of the Pol β protein was measured by immunoblotting 75 μg of total cellular proteins with the Pol β antibodies (Pr. S. Wilson, NIEHS, Research Triangle Park, NC) and the actin antibodies AC-40 (Sigma-Aldrich Chemical Co). Blots were developed by enhanced chemiluminescence chemiluminescent detection (Amersham).

Karyotype Analyses. Cell cultures were incubated in medium containing nocodazole (10 μM) for 3 h. Cells were harvested, incubated in hypotonic medium, and fixed with methanol, and the chromosomes were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma). Chromosomal distributions included the analysis of 100 metaphase spreads for each experiment, excluding polyploid cells.

Immunofluorescence Cell Staining. Cells were grown on glass coverslips in six-well plates at a density of 12,500 cells/cm^2. Later (24 h), they were centrifuged 5 min at 1000 rpm (Sigma 3K10; Bioblock Scientific) and fixed as described in protocol B (12), except that permeabilization was performed 45 s in buffer containing 0.5% Triton X-100. Then, cells were reacted for 1 h at 37°C and overnight at 4°C with R75 rabbit antibodies raised against the amino acid regions 434–451 of human γ-tubulin (13). Nuclei and chromosomes were stained with DAPI (0.2 μg/ml). For each cell line, ≥100 premetaphase or metaphase figures were analyzed.

Cell Cycle Analysis. Cells were seeded at 7,000 cells/cm^2 24 h before the addition of 1 μM nocodazole. After 6, 10, or 12 h of treatment, adhesion and floating cells were collected and washed twice with PBS. Cells were incubated for 10 min at 25°C in a buffer containing 3.4 mM trisodium citrate, 0.5 mM Tris, 0.1% NP40, 1.5 mM spermine tetrahydrochloride, and 30 μg/ml trypsin; RNase A and trypsin inhibitor were added for 10 min at 25°C in the same buffer to the final concentrations of 50 and 250 μg/ml, respectively. Labeling of DNA was performed at 4°C for 12 h in the previous buffer containing 140 μg/ml propidium iodide. Flow cytometric analysis (sup 25,000 cells/sample) was carried out using a FACscan (Becton Dickinson), and the percentage of

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[3] The abbreviations used are: Pol β, polymerase β; DAPI, 4′,6-diamidino-2-phenylindole; CHO, Chinese hamster ovary.

[4] Y. Canitrot, unpublished data.

cells in G_2-M phase was calculated using the ModFitLT software (Verity Software House). For evaluation of the mitotic index, cells were seeded at 12,500 cells/cm^2 at 24 h before the addition of 1 μM nocodazole. After 6, 10, or 12 h of treatment, adhesion and floating cells were collected, washed twice with PBS, and then resuspended in 1 ml of 3.7% formaldehyde in PBS, 10 min at 4°C. DAPI was added to a final concentration of 0.2 μg/ml and incubated 15 min at 37°C. After PBS washing, cells were resuspended in 50 μl, and 10 μl were dried and mounted in Mowiol. For each point, ≥1,000 cells were analyzed.

s.c. Tumorigenicity Assays. Cells were resuspended in 200 μl of PBS and injected with a 25-gauge needle into anesthetized 6–8-week-old immunodeficient athymic nude mice (BALB/c; Janvier Laboratory, Le-Genest, France). Tumor size was measured every 3–4 days. The time of initial tumor formation was defined as the time when the tumor had reached a diameter of 3 mm. Tumor volume was calculated with the formula 4/3r3. Tumor cells were reisolated by mincing the tumor, incubation in collagenase for 4 h, washing the cells in PBS, and replating the cells in minimum essential medium.

RESULTS AND DISCUSSION

A DNA expression vector harboring the cDNA encoding the rat DNA Pol β under the control of the herpes simplex virus thymidine kinase promoter was transfected into CHO cells to obtain isogenic strains overexpressing Pol β. In all clones that were tested, Pol β was 2–4-fold greater than control clones (CHO/AA8 parental cells and two independent CHO::Sh cell lines). To investigate the degree of aneuploidy, three independent transfected cells lines that overexpress Pol β by 4.5-, 3.2-, and 2.4-fold (CHO:Sh-pol β I, II, and III, respectively; Fig. 1*a*) were treated with nocodazole and subjected to karyotypic analysis. There was a significant difference between the chromosomal distributions of the Pol β-overexpressing cells and the control cell populations (Fig. 2). The three CHO::Sh-pol β clones accumulated an increased proportion of aneuploid cells compared with control CHO cells. Chromosome instability within each cell line consisted of chromosome losses as well as gains, indicating that deregulation of Pol β expression conferred a general chromosomal instability and that clonal selection attributable to loss or gain of specific chromosomes did not occur during culturing.

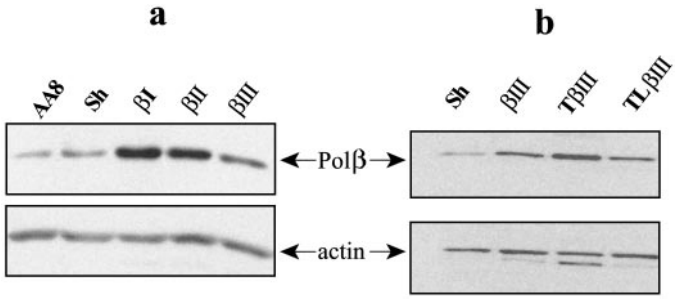

Fig. 1. Expression of Pol β in the CHO::Sh-pol β I (*βI*), CHO::Sh-pol β II (*βII*), and CHO::Sh-pol β III (*βIII*) cell lines as compared with control CHO-AA8 and CHO::Sh cells (cells transfected with the empty expression vector). In *a*, immunoblotting for the Pol β protein confirmed overexpression of the enzyme in the three cell lines. *b*, expression of Pol β in extracts from tumors formed in immunodeficient nude mice (*TβIII*) compared with the control CHO::Sh cells (*Sh*) and the CHO::Sh-pol β cells (*βIII*), as well as the cell line derived from the tumor (*TLβIII*). In this study, we used Pol β polyclonal antibodies that were provided by Dr. S Wilson, NIEHS.

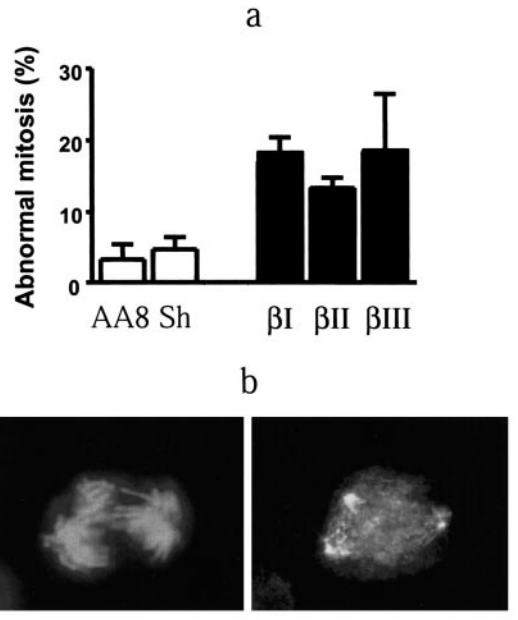

Fig. 3. Abnormal localization of γ-tubulin in CHO::Sh pol β during mitosis. *a*, cellular fraction showing an abnormal localization of γ-tubulin in both control (CHO/AA8 and CHO::Sh) and Pol β-overexpressing (CHO::sh-polβ I, CHO::sh-polβ II, and CHO::sh-polβ III) cell lines. Abnormal mitosis was characterized by an irregular distribution of nucleating material (number of γ-tubulin-staining structures > 2). Data represent three independent experiments. *b*, illustration of abnormal γ-tubulin distribution in CHO::sh-polβ III. *Left panel*, cells stained with DAPI for visualization of condensed chromosomes; *right panel*, γ-tubulin localization after labeling with anti-γ-tubulin antibodies.

Chromosome segregation is mediated by the mitotic spindle, which has a complex structural organization and precisely timed movements that ensure the accuracy of this process. In normal cells, the metaphase spindle is a bipolar structure containing microtubules that emanate from centrosomes at each pole with chromosomes aligned between the two half-spindles. γ−tubulin is associated with the centrosomes during all phases of the cell cycle and located at the pericentriolar material (14), which is involved in microtubule nucleation. Dysfunction of centrosome has been shown to be a frequent feature of malignant tumors and has been proposed to contribute to chromosome instability in cancers (15). Here, we hypothesized that aneuploidy observed in CHO::Sh-pol β cells may be a consequence of abnormal spindle formation. Therefore, we examined mitosis of these cells by immunostaining using anti-γ-tubulin antibodies. Quantification of the abnormal mitosis (γ-tubulin-staining structures > 2) showed that all Pol β-overexpressing cells displayed a significantly higher rate of abnormal mitosis compared with control cells (Fig. 3*a*). An example

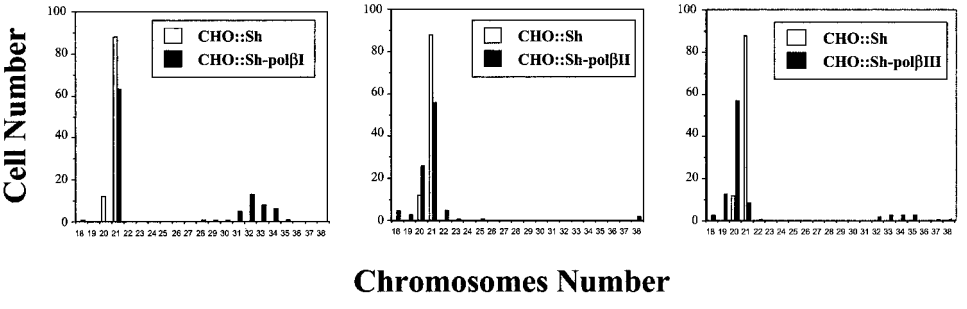

Fig. 2. Karyotypic analysis of Pol β-overexpressing cells. Cells were treated with the microtubule-disrupting agent nocodazole, and chromosomal distributions were analyzed on 100 metaphase spreads for each experiment.

of a typical abnormal mitosis is presented in Fig. 3*b*. These data indicate that amplification of centrosomes or fragmentation of nucleating material may occur in Pol β-overexpressing cells, processes which could result in deficient mitotic spindle and, therefore, in unequal chromosome segregation.

A mechanism of surveillance known as spindle checkpoint, which takes place during premetaphasic stages of mitosis, ensures the fidelity in chromosome transmission (16). A deficit in the activation of this checkpoint could result in a loss or gain of chromosomes. To assess the functionality of the spindle checkpoint in the Pol β-overexpressing cells as compared with the parental cells, we exposed cell cultures to nocodazole, and we analyzed the mitotic block of these cells at various times using flow cytometric analysis, as well as by scoring the mitotic index. Both experimental approaches indicated that the CHO::Sh-pol β cells accumulated at G_2-M or at mitosis at a lesser extent than control cells (Table 1). A statistical analysis confirmed that the two groups (controls and Pol β cells) differed significantly in their capacity to arrest after nocodazole treatment. We also observed numerous Pol β-induced multinucleated cells (data not shown), suggesting that inaccurate segregation of sister chromatids occurred and that reformation of nuclear membranes around groups of chromosomes took place. These findings reveal a Pol β-dependent defect in the mitotic checkpoint, and, because such inhibition has been demonstrated to result in abnormal number of chromosomes, it may participate to the aneuploid phenotype observed in the CHO::Sh-pol β.

The mechanistic explanations for the role of Pol β in centrosome aberrations and in the mitotic checkpoint deficit remain elusive. One possibility is that the probability for mutating the genes involved in centrosome duplication or organization and in the spindle checkpoint regulation could be enhanced by the overall nucleotide instability generated by high level of Pol β (3). There is increasing evidence for the interchangeability of DNA polymerases, and we (17) and others (18) proposed that mutation rates might be altered by varying the relative expression of error-prone polymerases. We demonstrated recently that excess of Pol β can substitute *in vitro* for the accurate replicative DNA polymerases during error-free DNA transactions, like Nucleotide Excision Repair (6) and DNA replication (19), rendering these processes mutagenic. However, we cannot rule out the possibility that high levels of Pol β may trap protein(s) or enzyme(s) engaged in genomic stability pathways, like recombination or repair. Lack of repair would leave unresolved damage in the genome, which may give rise to mechanical problems in segregating chromosomes or may trigger a signaling process affecting segregation. Recent reports demonstrated that cells lacking the RAD51-like genes *XRCC2* and *XRCC3*, which are involved in DNA repair by homologous recombination, or cells defective in BRCA1 or BRCA2 exhibited increased missegregation of chromosomes, as well as fragmentation of the centrosome (20–22). This potential trapping effect of Pol β would probably not affect base excision repair activity, because we observed previously that Pol β overexpression did not affect cell sensitivity to the alkylating agents methyl methanesulphonate and ethyl methane sulfonate, known to specifically introduce base damages repaired by BER (3).

Aneuploidy, which has been proposed to cause cancer, has been shown to be sufficient to explain genetic instability and the resulting karyotypic and phenotypic heterogeneity of cancer cells (8, 23). To investigate the tumor incidence that might arise from overexpression of Pol β, immunodeficient nude mice were inoculated with various control and transfected cell lines and then monitored for subsequent appearance of s.c. tumors (Table 2). Less than 25% of control inoculum with CHO/AA8 cells, a derivative from the CHO-K1 cell line that has mutant p53 sequence (24), as well as control CHO::Sh cells, induced formation of small tumors that developed slowly. In contrast, when CHO::Sh-pol β cells from three independent transfected clones (βI, βII, and βIII) were injected, 72% of them repeatedly and rapidly induced growing carcinomas, which were observable easily after 1 week. We concluded that excess Pol β provided CHO/AA8 cells a new phenotype that favors the proliferation of competent tumor cells. Tumor formation was also observed with low passage cells (passage 4; Table 2, last lane), suggesting that tumorigenic capability occurred early after ectopic expression of Pol β. Immunoblotting for the Pol β protein in tumor resulting from injection of the CHO::Sh-pol β III cells showed a 1.7-fold enhanced expression compared with the CHO::Sh-pol β III cell line before injection (Fig. 1*b*). An identical enhanced expression was observed, compared with the cell line that was derived from the tumor and growing *in vitro* (Fig. 1*b*). These observations provide evidence that high amounts of the polymerase may be important for tumor maintenance under the *in vivo* selective pressure.

Our data demonstrate the importance of a rigorous regulation of cellular expression of the error-prone DNA polymerases, such as Pol β for the maintenance of karyotypic stability. The inaccuracy itself of the enzyme may influence the cancer susceptibility we observed here, which corroborates with the recent findings revealing the importance of DNA polymerase δ proof-reading in reducing spontaneous tumor development in mice (25). Reduced fidelity might accelerate tumorigenesis by increasing the frequency of mutations that confer a selective growth advantage during cancer-cell evolution (1), like those affecting genes involved in centrosome organization or in the spindle checkpoint regulation.

Table 1 *Functionality of the spindle checkpoint in control and Pol β-overexpressing cells*

Control cells (CHO/AA8, CHO::Sh, and CHO::Sh::LacZ) and Pol β-overexpressing (CHO::sh-polβ I, CHO::sh-polβ II, and CHO::sh-polβ III) cell lines were cultured for the indicated time in the presence of nocodazole. The percentage of cells in the G_2-M and M phases of the cell cycle was estimated by flow cytometric analysis and scoring the mitotic index, respectively. For both experiments, a unilateral ANOVA test with two controlled factors was indicated by using the MINITAB® program. The *P versus* the group control is 0.006 and <0.01% in the G_2-M block and mitotic index analysis, respectively. These data are representative of three independent experiments.

	Cell lines	0 h	6 h	10 h	12 h
G_2-M block %	CHO/AA8	7.6	54.9	79.8	71.2
	CHO::Sh	11.9	65.8	80.0	61.0
	CHO::Sh::lacZ	11.9	72.0	87.7	94.1
	CHO::Sh-polβI	6.2	43.9	83.6	64.5
	CHO::Sh-polβII	5.9	55.0	70.4	61.1
	CHO::Sh-polβIII	11.0	47.3	70.8	54.5
Mitotic index %	CHO/AA8	3.1	39.0	55.5	45.0
	CHO::Sh	3.5	33.5	42.0	46.0
	CHO::Sh::lacZ	3.4	37.5	51.0	48.0
	CHO::Sh-polβI	4.2	28.0	41.0	33.0
	CHO::Sh-polβII	3.4	26.5	32.0	40.0
	CHO::Sh-polβIII	3.2	27.5	34.0	36.0

Table 2 *Formation of tumors in immunodeficient nude mice*

Cell lines	Pol β overexpression factor	No. of tumors/no. of injections (%)	
CHO/AA8	1	2/7 (28 %)	4/17 (23.5%)
CHO::Sh	1	2/10 (20%)	
CHO::Sh-polβI	8	5/6 (84 %)	21/29 (72.4%)
CHO::Sh-polβII	3	4/8 (50 %)	
CHO::Sh-polβIII	7	9/10 (90%)	
CHO::Sh-polβIII (Low passage)	7	3/5 (60 %)	

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