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Endogenous Factors Causative of Spontaneous DNA Damage that Leads to Random Integration in Human Cells

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Abstract

Random integration is a phenomenon in which transfected DNA molecules integrate into (random sites of) the host genome via non-homologous recombination. Although it is assumed that repair of DNA double-strand breaks leads to random integration events, how these endogenous DNA lesions are generated in living cells is poorly understood. In this study, we present evidence that DNA topoisomerase IIα (Top2α) and reactive oxygen species (ROS) are responsible for causing genomic DNA damage that leads to random integration. Specifically, we employed a human pre-B lymphocyte cell line to examine the effects of cellular Top2 expression levels and oxygen concentrations during cell culture. We find that treating cells with Top2α siRNA significantly reduces random integration frequency, while the absence of Top2β had little or no impact. We also show that cells continuously cultured under low (3%) oxygen culture conditions after electroporation display reduced random integration frequency compared to that under normal (21%) oxygen conditions. These findings support the notion that Top2α protein and ROS are endogenous factors that can produce DNA damage leading to random integration of transfected DNA in human cells.

Keywords: DNA damage; Random integration; Top2α; Top2β

Introduction

Mammalian cells possess the ability to perform nonhomologous recombination reactions, which require little or no sequence homology between DNA substrates [1,2]. Recent evidence indicates that the nonhomologous end-joining (NHEJ) pathway accounts for most, but not all, of nonhomologous recombination reactions occurring in the cell nucleus [3]. NHEJ and homologous recombination are the two major pathways for repairing DNA double-strand breaks (DSBs) that result from endogenous mechanisms as well as exposure to exogenous genotoxic agents [4]. A practical application of nonhomologous recombination is the generation of transfectants (i.e., random integrants) that stably express a transgene(s) of interest. Although the precise mechanism of random integration is not fully understood, it is believed that random integration results from non-homologous recombination-mediated repair, particularly NHEJ, of a spontaneous chromosomal DSB accidentally induced by endogenous factors [5-7]. In this study, we focused on DNA topoisomerase II (Top2) and reactive oxygen species (ROS) as the endogenous factors that induce DSBs causative of random integration.

Top2 is a ubiquitous nuclear enzyme that alters the topological structure of DNA and chromosomes through a transient DSB and subsequent religation of the DSB [8]. The enzyme has been implicated in many aspects of DNA metabolism, including DNA replication, transcription, and chromosome condensation/segregation [8]. Mammalian cells express two genetically distinct Top2 isoforms, α and β, which are differentially regulated and play different roles in living cells [8]. Top2α is most abundantly expressed in rapidly growing tissues and its expression is cell cycle-regulated, peaking in G_2/M, whereas the β-isoform is expressed in virtually all tissues and throughout the cell cycle [9-13]. Top2-targeting agents, such as etoposide, are among the most effective and widely used anticancer drugs in cancer chemotherapy [14]. These agents are referred to as "Top2 poisons", as they convert the essential enzyme into a highly cytotoxic DNA-damaging agent through the formation of "cleavage complex" (also called "cleavable complex"), in which a Top2-linked DNA strand-passing intermediate is stabilized, allowing the generation of a DSB [14]. It has been demonstrated that NHEJ plays a crucial role in the repair of Top2 inhibitor-induced DSBs [15,16]. Earlier work has shown that Top2 inhibitors can enhance random integration via nonhomologous recombination in mammalian cells [17,18]. Intriguingly, we have recently shown that inhibition of Top2α, not Top2β, is critical for the enhancement of random integration [19]. It remains to be elucidated, however, whether Top2α protein is actually involved in spontaneously occurring (i.e., Top2 inhibitor-independent) random integration events under physiological conditions, namely in the absence of forced poisoning of Top2 protein.

ROS are produced naturally as a product of oxidative metabolism in the mitochondrial electron transport chain and include superoxide anion radical, hydrogen peroxide and the hydroxyl free radical [20]. ROS are capable of inducing oxidative damage of DNA, including single-strand breaks and base and nucleotide modifications, such as 8-oxo-2'-deoxyguanine [21]. Intriguingly, ROS are also reported to generate chromosomal DSBs and that low oxygen culture conditions can reduce these DNA lesions [22]. We therefore reasoned that random integration frequency might be decreased if the transfected cells were cultured under low oxygen conditions.

In this report, we find using the human cell line Nalm-6 that treating cells with Top2α siRNA significantly reduces random integration frequency, thus providing direct evidence that spontaneous Top2α-induced DSBs indeed cause random integration in human cells. We also show that cells continuously cultured at 3% oxygen concentration (3% O_2/5% CO_2/92% N_2) display reduced random integration frequency compared to that at 21% oxygen concentration, providing the first direct evidence that random integration is decreased under low oxygen culture conditions. From these results, we propose that Top2α protein and ROS are endogenous factors that can produce

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DNA damage leading to random integration of transfected DNA in human cells.

Materials and Methods

Cells and culture conditions

The human pre-B cell line Nalm-6 and its derivatives were cultured in ES medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% calf serum (Hyclone, Logan, UT) and 50 μM 2-mercaptoethanol at 37°C in a humidified atmosphere containing 5% CO_2 [23]. *TOP2A*$^{+/-}$ and *TOP2B*$^{-/-}$ cells were created by gene targeting, as reported previously [24] (Figure S1). A *TOP2B*$^{-/-}$*TOP2A*$^{+/-}$ cell line was created by gene targeting (heterozygous disruption) of the *TOP2A* gene using the *TOP2B*$^{-/-}$ cells, as described previously (Figure S1).

Drugs

Etoposide was purchased from BioVision (100 mM;Mountain View, CA) and dissolved in dimethyl sulfoxide. Bleomycin was purchased from Wako (Osaka, Japan) and dissolved in distilled water to a concentration of 1 mg/ml. These drugs were stored frozen in aliquots at -20°C.

Transfection and integration assays

Random integration assays were carried out using Nalm-6 cells, essentially as described [7,19]. Briefly, 4×10^6 cells were electroporated with 4 μg of NotI-linearized pLucPuro per 40-μl cuvette of Electro Gene Transfer Equipment (GTE-1; Shimadzu, Kyoto, Japan). After 15 min, cells were transferred into growth medium with or without drugs, and cultured for 24 hr at 21% or 3% oxygen concentration. Transfected cells were then collected, counted and plated into agarose medium containing 0.5 μg/ml puromycin (Wako). Meanwhile, small aliquots of transfected cells were plated into drug-free agarose medium to determine the plating efficiency. After two-week cultivation, the integration frequency was calculated by dividing the number of puromycin-resistant colonies by that of cells plated multiplied by the plating efficiency. pLucPuro was constructed by subcloning a SalI fragment containing a PGK promoter, a puromycin-resistance gene and polyA signal sequences from pPGKPuro into SalI-digested pGL4-13 (Figure S2).

Small interfering RNA (siRNA)

Top2α-targeting siRNA (sense,5'-AAAAGACUGUCU-GUUGAAAGAdTdT-3'; antisense,5'-UCUUUCAACAGACAGU-CUUUUdTdT-3') corresponding to nucleotides 76 to 96 relative to the first nucleotide of the start codon was purchased from Qiagen (Tokyo, Japan) [24]. The siRNA was diluted with RNase-free water and stored frozen at -20°C. Log-phase cells were electroporated with 200 pmol of siRNA alone or along with NotI-linearized pLuc Puro, followed by western blot analysis or random integration assays.

Luciferase assays

Luciferase assays were performed as described previously [19]. Briefly, after electroporation with pLucPuro, cells were cultured in growth medium for 5 hr. An aliquot (3×10^4 cells) was then suspended in 50 μl of growth medium, and subjected to luciferase assays using the ONE-Glo Luciferase Assay System (Promega, WI) according to the manufacturer's instructions.

Western blot analysis

Cells were suspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 10% glycerol, 2 mM DTT, 150 mM KCl, and protease inhibitor cocktails (Sigma-Aldrich, St Louis, MO)), sonicated three times for 5 sec, and centrifuged at 15,000 rpm for 30 min at 4°C. Lysates corresponding to 20 μg were mixed with 2xSDS sample buffer, and heated at 95°C for 2 min, and subjected to electrophoresis on a 0.1% SDS-6% polyacrylamide gel. Polypeptides were transferred onto a PVDF membrane in a semidry-type blotting apparatus for 1 hr at 100 mA. Molecular masses of polypeptides were estimated by comparison with migration of Precision plus protein all blue standards (Bio-Rad, Hercules, CA). Membranes were first blocked with 5% non-fat milk powder in Tris-buffered saline containing 0.05% Tween 20. The antibodies used were mouse monoclonal antibody against human Top2α or Top2β, mouse anti-human Ku70 monoclonal antibody (1:500; BD Transduction Laboratories, Bedford, MA) and mouse anti-human actin monoclonal antibody (1:2000; Sigma-Aldrich). Levels of expression were quantified using a Fuji Image analyzer LAS-1000UV mini and a MultiGauge software (Fuji Film, Tokyo, Japan) [24].

Results

Top2α siRNA-treated cells exhibit reduced random integration frequencies

As mammalian cells express two genetically distinct isoforms of Top2, we first analyzed the contribution of each isoform to random integration. Specifically, we examined the random integration frequency in Nalm-6 wild-type cells (proficient for Top2α and Top2β), *TOP2A*$^{+/-}$ (heterozygously null for Top2α) and *TOP2B*$^{-/-}$cells (homozygously null for Top2β) (Figure S1). As shown in Figure 1A, the random integration frequency of pLucPuro plasmid in these cells was comparable to that in wild-type cells. Transient expression analysis revealed no significant change in luciferase activity (Figure 1B). These data suggest that neither heterozygous disruption of *TOP2A* nor homozygous disruption of TOP2B has little or no effect on the frequency of random integration. We further examined the contribution of Top2α by constructing a *TOP2B*$^{-/-}$*TOP2A*$^{+/-}$ cell line by gene targeting (Figure S1), which led us find that the heterozygous disruption of *TOP2A* does not affect the random integration frequency even in the complete absence of Top2β (Figure 1A).

As the human *TOP2A* gene is essential for cell proliferation [25,26], we next employed Top2α siRNA to analyze the random integration frequency in cells with greatly reduced Top2α levels. Western blot

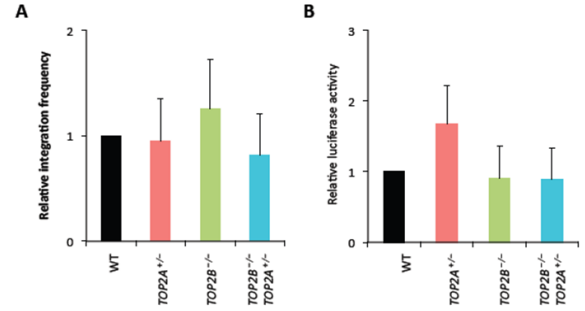

Figure 1: *TOP2* mutants retain wild-type levels of random integration frequency. (A, B) Wild-type cells and *TOP2* mutants were electroporated with pLucPuro and subjected to random integration assay (A) or luciferase assay (B). The random integration frequency and the luciferase activity in wild-type cells were taken as 1, and the relative integration frequency or luciferase activity in *TOP2* mutants was calculated. Data are the mean ± S.D. of at least three independent experiments.

analysis showed that Top2α expression level in Top2α siRNA-treated cells was reduced ~90% compared to mock-treated cells (Figure 2A and 2B). As shown in Figure 2C, the random integration frequency in Top2α-knockdown cells was reduced ~40%. Luciferase activity in Top2α-knockdown cells was comparable to that in mock-treated cells (Figure 2D). These results suggest that random integration frequency is reduced when Top2α expression is strongly suppressed. This is in sharp contrast to our previous finding that absence of Top2β had little or no effect on the frequency of random integration [19].

Random-integration enhancement by Top2 inhibitor is Top2α dependent

We next examined the effect of DSBs induced by the Top2 inhibitor etoposide on random integration frequency. Etoposide targets both isoforms of Top2 in a cell [14]. As shown in Figure 3A, random integration frequency was increased in all the cell lines in a drug concentration-dependent manner. Importantly, the level of enhancement was less prominent in *TOP2A*$^{+/-}$ and *TOP2B*$^{-/-}$*TOP2A*$^{+/-}$ cells. In contrast, random integration frequency in *TOP2B*$^{-/-}$ cells was comparable to that observed in wild-type cells. Very similar results were obtained using NK314, a Top2α-specific poison [24] (data not shown). These results suggest that the repair process for Top2α-mediated, not Top2β-mediated, DSBs is involved in Top2 poison-induced random integration.

We also examined the contribution of Top2 expression levels to bleomycin-enhanced random integration. Bleomycin binds transition metals, Fe(II) or Cu(I), and oxygen and, in the presence of a one-electron reductant, can catalyze formation of single-stranded and double-stranded DNA lesions [27]. We have previously shown that, similar to Top2 inhibitors, bleomycin can enhance random integration in human somatic cells [28]. As expected, bleomycin treatment of cells greatly enhanced random integration (Figure 3B) and, unlike the case of etoposide, the level of enhance mention *TOP2A*$^{+/-}$ and *TOP2B*$^{-/-}$ *TOP2A*$^{+/-}$ cells was comparable to that in wild-type cells. These results suggest that Top2α does not participate in bleomycin-enhanced random integration, while it is specifically involved in Top2 poison-induced random integration.

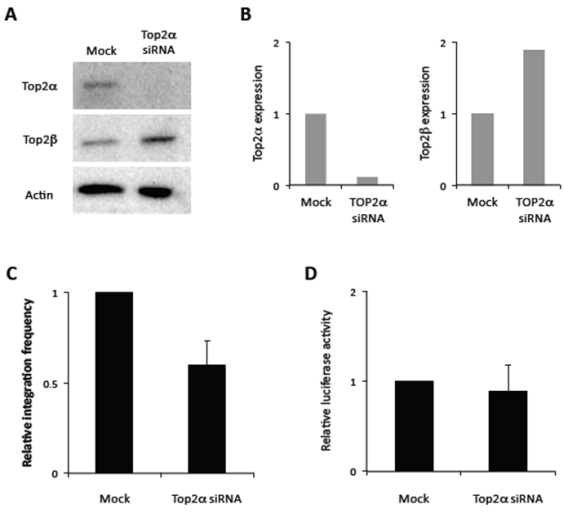

Figure 2: The random integration frequency in Top2α siRNA-treated cells is reduced. (A,B) Western blot analysis for Top2 in Top2α siRNA-treated cells. Actin served as a loading control. (B) Relative expression levels of Top2 proteins in Top2α siRNA-treated cells. (C,D) Mock-treated cells and Top2α siRNA-treated cells were electroporated with linearized pLucPuro and subjected to random integration assay (C) or luciferase assay (D). The random integration frequency or the luciferase activity in mock-treated cells was taken as 1, and the relative integration frequency or luciferase activity in Top2α siRNA-treated cells was calculated. Data are the mean ± S.D. of three independent experiments.

Low oxygen culture conditions reduce the frequency of random integration

The above data revealed that Top2α participates in spontaneously occurring random integration as well as Top2 inhibitor-induced random integration. Apparently, however, other endogenous factors should also be involved in causing DNA damage that leads to random integration events. Thus, we next focused on endogenous free radicals, ROS, as the factor that induces spontaneous chromosome breaks. For this purpose, we cultured transfected cells for 24 hr under normal (21%) or low (3%) oxygen conditions, and performed colony formation under normal or low oxygen conditions. More specifically, we examined the random integration frequency by culturing cells under either of the following culture conditions (Figure 4A): (i) cells were cultured

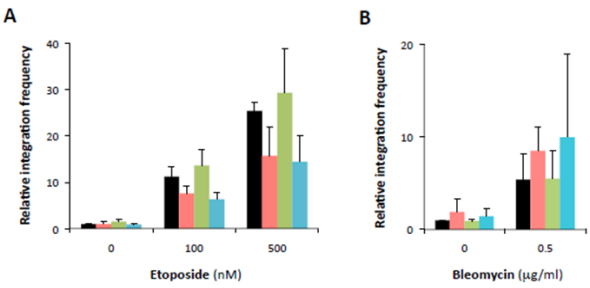

Figure 3: Random-integration enhancement by etoposide is less prominent in *TOP2A*$^{+/-}$ cells. (A,B) Wild-type cells and *TOP2* mutants were electroporated with linearized pLucPuro, and treated with the indicated concentrations of etoposide (A) or bleomycin (B) for 24 hr. The random integration frequency in wild-type cells was taken as 1, and the relative integration frequency in *TOP2* mutants was calculated. Data are the mean ± S.D. of three independent experiments. Symbols are as in Figure 1.

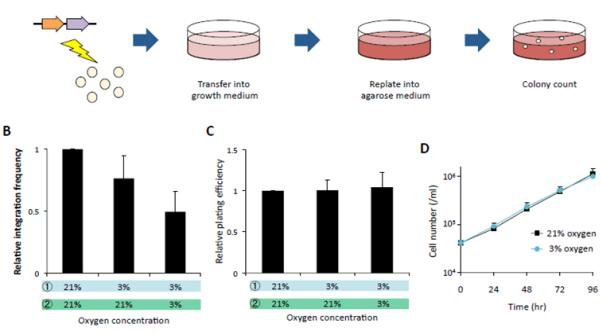

Figure 4: The 3% oxygen condition reduces the frequency of random integration. (A) Strategy of random integration assay. Nalm-6 cells (4x10^6) were electroporated with 4 μg of linearized plasmid vector and cultured for 24 hr at 21% or 3% oxygen concentration, and then allowed for colony formation at 21% or 3% oxygen concentration. (B) Relative integration frequency at the indicated oxygen conditions. Cells were electroporated with linearized pLucPuro, and incubated at the indicated oxygen concentration. Data are the mean ± S.D. of three independent experiments. (C) Plating efficiencies of cells cultured under the indicated oxygen conditions after electroporation. Data are the mean ± S.D. of three independent experiments. (D) Growth curves of cells cultured at 21% or 3% oxygen concentration. Data are the mean ± S.D. of three independent experiments. Where absent, error bars fall within symbols.

continuously under normal oxygen conditions after electroporation, (ii) cells were cultured under low oxygen conditions during cultivation after electroporation, and then allowed for colony formation under normal oxygen conditions, or (iii) cells were continuously cultured under low oxygen conditions after electroporation.

As shown in Figure 4B, we found that low oxygen concentration did reduce the frequency of random integration. In particular, random integration frequency was decreased by half in cells continuously cultured under low oxygen conditions after electroporation. It should be noted that the plating efficiency and the growth rate of cells under low oxygen conditions were indistinguishable from that under normal oxygen conditions, indicating that low oxygen culture conditions do not disturb cell proliferation *per se* (Figure 4C and 4D).

Discussion

It has been generally assumed that random integration of foreign DNA results from the repair of DNA damage, specifically spontaneous chromosomal DSBs that are caused by endogenous factors. However, it is largely unknown how these DSBs are generated in the cell nucleus. In this study, we focused on cellular Top2 proteins (α and β) and free radicals as the factors responsible for endogenous DSBs, and examined their contribution to random integration frequency. The data presented here clearly indicate that Top2α participates in spontaneously occurring random integration as well as Top2 inhibitor-induced random integration. Similarly importantly, our data provide the first evidence that oxygen concentration during cell culture does affect the frequency of random integration in human cells.

Our observation that Top2α siRNA-treated cells display reduced random integration frequency provides evidence that spontaneously occurring Top2α-mediated DSBs can cause random integration. We also found that inhibition of Top2α not Top2β, is important for etoposide-induced random integration. Why are Top2α-mediated DSBs specifically involved in random integration? Earlier work has identified mammalian cell lines that do not express Top2β, and indeed Top2β-null cells do not exhibit growth defects at the cellular level [24,29-32]. Thus, it seems quite reasonable that only Top2α is responsible for causing random integration, while Top2β is not. However, we reported previously that Nalm-6 cells express similar levels of Top2α and Top2β [33], which does not account for the Top2α dominance in causing random integration. One possibility might be a cell cycle dependency of Top2α-mediated DSBs. Top2α expression is cell cycle-regulated, peaking in G_2/M, whereas the β-isoform is expressed throughout the cell cycle [9-11]. Possibly, Top2α-mediated DSBs that arise in G_2/M phase might be convenient for random integration to occur. Interestingly, Cowell et al. [34] have recently reported that *TOP2B*$^{-/-}$ Nalm-6 cells have roughly equal amounts of chromosomal DSBs after etoposide treatment when compared to wild-type Nalm-6 cells. This finding clearly indicates that Top2β does not play a major role in producing etoposide-induced DSBs, a notion that well explains our observations described herein. It should be noted, however, that Top2β-mediated DSBs, albeit fewer in number, are actually induced by etoposide treatment, and this type of DNA damage governs the genotoxicity of drug-treated cells [34]. Unlike Top2α, Top2β is thought to be specifically required for transcription, and thus binds to promoter regions in the genome [35,36]. Possibly, Top2β-mediated DSBs, if any, might be strictly regulated not to use NHEJ for repair, in a manner that does not permit genomic incorporation of foreign DNA. This idea may well account for the inertness of Top2β in causing random integration.

We have also shown in this study that culturing cells under low oxygen conditions results in a reduced random integration frequency. It is assumed that low oxygen culture conditions should reduce cellular free radicals, resulting in a decrease in the amount of spontaneous DNA damage (strand breaks as well as oxidative damage) in the genome. Indeed, Karanjawala et al. [22] have reported that the number of spontaneous DSBs due to ROS produced within a cell is substantial and that oxygen metabolism actually causes chromosomal DSBs that likely rely on NHEJ for repair. More specifically, cells lacking DNA ligase IV (the critical DNA ligase for NHEJ to be completed) had increased levels of ROS [22]. Karanjawala et al. [22] further showed that low oxygen (3%) culture conditions could reduce those DSBs. These findings are consistent with our observation that the random integration frequency decreases in cells cultured under low oxygen conditions, due to a reduction of ROS induced-DSBs in the cell. It should be noted that the fact that the low oxygen culture condition is effective in reducing genomic DNA damage as well as unwanted random integration of foreign DNA might have implications for future gene/cell therapy. For example, iPS cells have been shown to be more stably maintained under low oxygen culture conditions ([37] and our unpublished observations). Even more importantly, as random integration is a major obstacle in gene targeting via homologous recombination, a decreased random integration frequency should be preferable to increasing the frequency of targeted gene inactivation/correction [7]. It is therefore expected that artificial manipulation of random integration will contribute to significant improvements in gene-targeting technology.

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