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UV-induced mutations in epidermal cells of mice defective in DNA polymerase η and/or ι

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ABSTRACT

Xeroderma pigmentosum variant (XP-V) is a human rare inherited recessive disease, predisposed to sunlight-induced skin cancer, which is caused by deficiency in DNA polymerase η (Polη). Polη catalyzes accurate translesion synthesis (TLS) past pyrimidine dimers, the most prominent UV-induced lesions. DNA polymerase ι (Polι) is a paralog of Polη that has been suggested to participate in TLS past UV-induced lesions, but its function *in vivo* remains uncertain. We have previously reported that Polη-deficient and Polη/Polι double-deficient mice showed increased susceptibility to UV-induced carcinogenesis. Here, we investigated UV-induced mutation frequencies and spectra in the epidermal cells of Polη- and/or Polι-deficient mice. While Polη-deficient mice showed significantly higher UV-induced mutation frequencies than wild-type mice, Polι deficiency did not influence the frequencies in the presence of Polη. Interestingly, the frequencies in Polη/Polι double-deficient mice were statistically lower than those in Polη-deficient mice, although they were still higher than those of wild-type mice. Sequence analysis revealed that most of the UV-induced mutations in Polη-deficient and Polη/Polι double-deficient mice were base substitutions at dipyrimidine sites. An increase in UV-induced mutations at both G:C and A:T pairs associated with Polη deficiency suggests that Polη contributes to accurate TLS past both thymine- and cytosine-containing dimers *in vivo*. A significant decrease in G:C to A:T transition in Polη/Polι double-deficient mice when compared with Polη-deficient mice suggests that Polι is involved in error-prone TLS past cytosine-containing dimers when Polη is inactivated.

1. Introduction

Xeroderma pigmentosum (XP) is a genetic disorder characterized by ultraviolet light (UV) sensitivity and increased incidence of skin cancers. XP has been classified into eight genetic complementation groups, XP-A–G and XP-V [1]. Cells derived from XP-A through XP-G patients are deficient in nucleotide excision repair (NER), which repairs a variety of DNA lesions including UV-induced cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts. The variant form (XP-V) is characterized by proficiency in NER but deficiency in translesion synthesis (TLS). TLS is a mechanism that prevents replication blockage at a DNA lesion by using specialized DNA polymerases that incorporate nucleotides opposite the lesion and continue DNA synthesis past the site of damage [1]. We have identified human DNA polymerase η (Polη) as the product of the gene associated with XP-V, *POLH*. Polη catalyzes accurate translesion synthesis (TLS) past *cis–syn* thymine–thymine dimers [2–5], an important mechanism to prevent UV-induced skin cancers in human cells.

Mammalian cells are now known to have fifteen DNA template-dependent DNA polymerases, and these are classified into five families, A, B, X, Y, and AEP (*a*rchaeo-*e*ukaryotic *p*rimase superfamily)

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according to similarities in their primary structures [6–9]. The Y-family, which was shown to carry out TLS past some lesions, consists of Polη, Polι, Polκ, and REV1 [10]. Polι, encoded by the *POLI* gene, incorporates one or two nucleotides opposite (6-4) photoproducts *in vitro* but cannot bypass these lesions by itself [11,12], although limited Polι dependent bypass of CPD lesions has been observed depending upon the local sequence context and metal ion used as a cofactor [11,13–15]. Whereas Polι is a paralog of Polη, no human disease related to Polι-deficiency has been identified so far, and its physiological relevance has not been clarified. Polκ is the eukaryotic homologue of the *Escherichia coli* DinB protein (DNA polymerase IV) and has been shown to be involved in accurate TLS past N^2-adducts of dG and also in both spontaneous and induced mutagenesis [16,17]. Although Polκ was suggested to be involved in NER of UV-induced lesions [18,19], it seems unlikely that Polκ plays an important role in TLS past UV lesions since the enzyme showed no activity to bypass past CPD and (6-4) photoproduct *in vitro* [20]. REV1 has a dCMP transferase activity, but the protein appears to exert its role in UV-induced responses, as a scaffold protein interacting with Polζ, Polη, Polι, and Polκ [10]. Polζ is a B-family polymerase containing REV3 and REV7 subunits and is believed to function as an "extender" enzyme after a TLS DNA polymerase (for example, Polι) inserting a nucleotide opposite a given DNA lesion [10].

To investigate the physiological roles of Polη and Polι, we have previously generated mice mutated in *Polh* and/or *Poli* genes [21]. Polη-deficient mice were generated by inserting a G418 resistance gene cassette into exon 8 of the *Polh* gene, resulting in production of a truncated, nonfunctional Polη protein. Using the 129 mouse derived embryonic stem (ES) cell line, which carries a spontaneous Polι nonsense mutation [22], we obtained Polη/Polι double-deficient mice as well as Polι-deficient mice. We found that the incidence of skin tumors was greatly increased in the Polη- and Polη/Polι double-deficient mice after UV irradiation, that Polη/Polι double-deficient mice started to develop skin tumors earlier than Polη-deficient mice, and that the average number of skin tumors was higher in the double-deficient mice than in the Polη-deficient mice. In addition, we found that epithelial and mesenchymal tumors were formed in Polη- and Polι-deficient mice, respectively [21]. These results suggest that, in addition to Polη, Polι also participates in suppressing skin carcinogenesis. Other groups had also shown the importance of Polη and Polι to suppress UV-induced skin tumors. Polη-deficient mice, generated by disruption of exon 4 of the *Polh* gene, are highly susceptible to UV-induced skin tumors [23]. Polη/Polι double-deficient mice, in turn, develop UV-induced skin tumors earlier than Polη-deficient mice [24].

To investigate the functions of Polη and Polι in suppressing UV-induced carcinogenesis, we examined the mutation frequencies and spectra in UV-irradiated and unirradiated epidermis in Polη-deficient, Polι-deficient, and Polη/Polι double-deficient mice, using the *rpsL* transgene as a mutation reporter sequence. Our results show that Polη plays crucial roles in suppressing any types of UV-induced base substitution at dipyrimidine sites *in vivo* and that Polι participates in error-prone TLS past UV lesions in the absence of Polη.

2. Materials and methods

2.1. Generation of transgenic mice

The *rpsL* transgenic mouse line (ssw2-14p) used in this study was derived from C57BL/6J mice, but carries approximately 100 hemizygous copies of the pSSW plasmid; it has been described previously [25]. The pSSW plasmid carries a reporter gene, *rpsL* from *E. coli*, with a dominant streptomycin-sensitive (Sm^s) phenotype in bacterial cells, and a kanamycin-resistant (Km^r) gene. Polη- and/or Polι-deficient mice were established as previously described [21]. *Polh*$^{+/-}$, *Poli*$^{+/-}$ mice were mated with the *rpsL* transgenic mice to generate *rpsL*$^{Tg/+}$ *Polh*$^{+/-}$ *Poli*$^{+/-}$ mice. By mating the *rpsL*$^{Tg/+}$ *Polh*$^{+/-}$ *Poli*$^{+/-}$ mice with *Polh*$^{+/-}$ *Poli*$^{+/-}$ mice, *rpsL*$^{Tg/+}$ *Polh*$^{-/-}$ *Poli*$^{+/+}$, *rpsL*$^{Tg/+}$ *Polh*$^{+/+}$ *Poli*$^{-/-}$, and *rpsL*$^{Tg/+}$ *Polh*$^{-/-}$ *Poli*$^{-/-}$ mice were generated. The handling and sacrifice of all animals were carried out in accordance with nationally prescribed guidelines, and ethical approval for the studies was granted by the Committee for Animal Experiments, Graduate School of Frontier Biosciences of Osaka University.

2.2. Preparation of UVB irradiated and unirradiated epidermal genomes

At 8 weeks of age, after shaving of dorsal hair, mice from each respective genotype were irradiated with UVB (FL20SE-E; Toshiba, Tokyo, Japan) at 400 J/m^2. One week after irradiation, these mice were sacrificed, and the irradiated dorsal and unirradiated ventral skins were collected. These skin samples were treated with 20 mM EDTA in PBS at 37 °C, and the epidermis was peeled from the dermis using a spatula. The isolated epidermal samples were frozen in liquid nitrogen and stored at −80 °C. To prepare genomic DNA, the epidermal samples were first incubated in lysis buffer (10 mM Tris–HCl (pH 8.0), 100 mM EDTA, 200 mM NaCl, 0.5% SDS, 0.1 mg/ml RNaseA) at 37 °C for 1 h, followed by 1 mg/ml Proteinase K for 3 h. After phenol/chloroform extraction, genomic DNA was obtained by ethanol precipitation.

2.3. rpsL mutagenesis assay

Mutation analyses were performed as described [25] with minor modifications. Briefly, 10 μg of isolated genomic DNA was digested with 35 U of *Ban*II (TaKaRa, Shiga, Japan) at 37 °C for 3 h, which incises once the pSSW plasmid, in order to excise the integrated shuttle vector at unit size. The *Ban*II digested DNA was then treated with 350 U of T4 DNA ligase (TaKaRa, Shiga, Japan) for 2 h at 16 °C for self-circularization. One microgram of the resulting DNA was introduced into *E. coli* DH10B cells by electroporation (1.8 kV/mm). Then, a portion of the cells were plated onto LB plates containing kanamycin (50 μg/ml), and the remainders were plated onto plates containing both kanamycin and streptomycin (200 μg/ml). These plates were incubated at 28 °C for 48 h. Total numbers of kanamycin-resistant colonies were calculated from the number of colonies formed and the amount of DNA used. Mutation frequency was calculated as the ratio of total colony numbers on the plate containing kanamycin and streptomycin to those on the plate containing kanamycin only. For mutation spectrum analysis, DNA fragments containing the *rpsL* gene were amplified by PCR from the kanamycin- and streptomycin-resistant colonies. The primers F (5′-CACCTGATTGCCCGACATTA-3′) and R (5′-CAGGGTCGGAACAGGAGAGC-3′) were used for the PCR. The amplified PCR products were directly sequenced with the forward primer (5′-GACGAATTCCCGGTTTGACTGGTC-3′) and the reverse primer (5′-GGATTGTCCAAAACTCTACGAG-3′). Mutations found in the *rpsL* coding region and surrounding sequences containing the promoter and ribosomal recognition regions (from position −120 to position 375) were exhibited in the mutation spectrum.

2.4. Statistical analyses

Statistical analyses for mutation frequency included the Student's *t*-test and Fisher's exact probability test. The CLUSTERM

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