Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo

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Introduction

Genetic polymorphisms in DNA repair genes have been considered to be genetic factors underlying cancer risk by causing inter-individual differences in the capacity to prevent mutagenesis by DNA damages (1). The OGG1 gene encodes a protein with DNA glycosylase and AP lyase activities that removes 8-hydroxyguanine (8OHG), an oxidatively damaged promutagenic base. The OGG1 protein has an ability to suppress mutagenesis of human cells; supporting the in vivo results of recent association studies that OGG1-Cys326 is a risk allele for several types of human cancers such as lung cancer, esophageal cancer, prostate cancer, orolaryngeal cancer and nasopharyngeal cancer (10;12; others reviewed in ref. 1). Consistent with these reports, in a recent case control study, the mean OGG1 activity in protein extracts from peripheral blood mononuclear cells in lung cancer patients was shown as being significantly lower than the activity in the control participants (13). Differential mutation suppressive ability was suggested between the OGG1-Ser326 and OGG1-Cys326 proteins, although this polymorphic amino acid is located outside the domains conserved among DNA glycosylases. The OGG1-Cys326 protein was shown to have a lower ability than the OGG1-Ser326 protein to suppress spontaneous mutations in an Escherichia coli (mutM mutY) strain that is defective in 8OHG repair (9). OGG1-Cys326 protein, expressed in and purified from bacterial cells, was shown to have slightly lower glycosylase activity in vitro than OGG1-Ser326 (14). Thus, it has been predicted that the OGG1-Cys326 allele confers cancer susceptibility due to its encoding the OGG1-Cys326 protein with a lower ability to prevent mutagenesis by 8OHG than the OGG1-Ser326 protein. However, in human cells, mutation suppressive activity against 8OHG has been examined only for the OGG1-Ser326 protein but not for the OGG1-Cys326 protein (4,5), and thus, the functional difference between the two polymorphic OGG1 proteins in human cells in vivo still remained unclear. Therefore, in this study, suppressive ability against 8OHG-induced G:C to T:A transversions was compared between the two polymorphic OGG1 proteins in human cells in vivo. For this purpose, we undertook a supF forward mutation assay employing a shuttle plasmid, pMY189-8OHG, containing a single 8OHG residue (4). pMY189-8OHG was transfected into H1299 human lung cancer cells, in which the endogenous OGG1 protein was not detectable by western blot analysis (4). Various amounts of each polymorphic OGG1 protein were transduced in H1299 cells through adenoviral vectors by infecting viruses at several MOIs (multiplicity of infection). G:C to T:A mutation frequency at the 8OHG site was estimated by a quantitative real-time PCR (QRT-PCR) method so that mutation frequencies in a large number of samples could be assessed rapidly and accurately. The results indicated that the OGG1-Cys326 protein has a lower ability to suppress mutations than the OGG1-Ser326 protein in human cells in vivo.

Materials and methods

Assessment of mutation frequency by QRT-PCR

Test (supF-159T) and reference (pBR327-ori) loci were amplified in a single tube by PCR in the presence of TaqMan probes for the test and reference loci, respectively. The reaction mixture of 25 μl in volume contained 12.5 μl of

Abbreviations: CI, confidence interval; 8OHG, 8-hydroxyguanine; MOI, multiplicity of infection; QRT-PCR, quantitative real-time PCR; WCE, whole cell extract.

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Preparation of adenoviruses to express polymorphic OGG1 proteins based on the averaged subcloned into the pcDNA3 plasmid (15; unpublished data). The OGG1-Full-length cDNAs for the OGG1-Ser326 and -Cys326 type 1a transcripts were PCR run included a seven-point standard (i.e. a serial dilution of mutant curve. Ct (threshold cycle) values were determined for the test and reference in the RPMI-1640 medium with 10% fetal bovine serum. H1299 is wild-type assessment of for OGG1-Gln249 using a Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). A cDNA fragment covering the coding sequence nt 1306, Genbank AB000410) for each polymorphic/mutant OGG1 was estimated as ng/µg of WCE. The protein amount of WCE loaded on each lane was calculated based on the signal intensity of α-tubulin on the same blot. Based on these data, the amount of exogenous OGG1 protein in each sample was estimated as ng/µg of WCE.

Statistics

Regression lines were estimated by the least squares method using StatView 5.0 (SAS Institute, Cary, NC).

Results and discussion

Assessment of G:C to T:A mutation frequencies by a QRT-PCR method

We demonstrated previously that the mutation frequency in the supF gene of the pMY189-8OHG plasmid was 5 × 10⁻² in H1299 cells after 48 h of transfection, and that G:C to T:A transversion was specifically (> 95%) induced at position 159 of the supF gene where a 8OHG:C base pair was incorporated (5). Thus, to assess the frequency of G:C to T:A mutation at position 159 of the supF gene in the pMY189-8OHG plasmid more easily and rapidly with a wide range, we developed a QRT-PCR assay, in which the copy number of mutant supF fragments relative to that of total plasmid was considered as being the frequency of G:C to T:A mutations at position 159 of the supF gene in the pMY189-8OHG plasmid. In this assay, to selectively amplify the mutant plasmids with T:A at position 159, a mutation-specific forward primer with T at the 3' end instead of G was prepared. Then a mutant supF fragment of 138 bp and a plasmid backbone (pBR327-ori) fragment of 285 bp were designed to be amplified simultaneously in a single PCR (Figure 1A).

To determine the sensitivity and accuracy of this assay system, we first performed QRT-PCR using mixtures of mutant pMY189 plasmid (with T at position 159) and wild-type pMY189 plasmid (with G at position 159) as templates.

SupF forward mutation assay

The pMY189-8OHG plasmid, containing a single 8OHG-cytosine pair at nucleotide position 159 of the supF gene, was prepared according to the method described previously (4,5). H1299, a lung cancer cell line, was cultured in the RPMI-1640 medium with 10% fetal bovine serum. H1299 is wild-type for the OGG1 gene, however, the endogenous OGG1 protein is not detectable in H1299 by western blot analysis using anti-OGG1 antibody (4,5). For the assessment of in vivo mutation frequency, 5 × 10⁴ of H1299 cells were seeded on a 100-mm tissue culture plate and infected with adenovirus after 24 h. After another 24 h, half (~2.5 × 10⁴ cells) of the cells was subjected to western blot analysis, and the other half of the cells was transfected with 1 µg of pMY189-8OHG plasmid using DMRIE-C reagent (Invitrogen, Carlsbad, CA) (16). After 7–9 days, cells were collected and destroyed by freezing and thawing. Harvested adenoviruses were further propagated in HEK293, purified by CsCl density gradient centrifugation, and were quantified by optical absorbance (17).

Adenoviruses were expressed to detect the OGG1 protein, the WCE of uninfected H1299 cells for standardizing the amounts of protein loaded. Cell extracts were incubated with 5 µg/ml of a mouse anti-human OGG1 antibody (mAb-7E2) (IBL, Fujioka, Japan), followed by exposure to a 1:5000 dilution of a sheep anti-mouse Ig-HRP linked F(ab')₂ fragment (Amersham Biosciences, Tokyo, Japan). Proteins were visualized by using the Supersignal West Pico (Pierce, Rockford, IL) and quantified by an ATTO CS analyzer (Atto, Tokyo, Japan). After detection of the OGG1 protein, the antibodies were stripped and the membranes were subjected to the detection of α-tubulin using 1 µg/ml of a mouse anti-α-tubulin antibody (Oncogene Research Products, San Diego, CA). The amount of exogenous OGG1 protein in each lane was calculated based on the signal intensity of α-tubulin on the same blot. The protein amount of WCE loaded on each lane was standardized based on the signal intensity of α-tubulin on the same blot. Based on these data, the amount of exogenous OGG1 protein in each sample was estimated as ng/µg of WCE.

Western blot analysis

Fig. 1. Estimation of 8OHG-induced mutation frequency by QRT-PCR. (A) Locations of primers, TaqMan probes and the site of 8OHG in the pMY189 plasmid. The primer to selectively amplify a supF fragment with T:A at position 159 is shown as a filled arrow, while others are shown as open arrows. TaqMan probes are shown as thick bars. (B) QRT-PCR amplification of a supF fragment with T:A at position 159. The results of 1 ng (circle) or 100 pg (square) of templates are shown. Data represent mean values estimated by QRT-PCR in triplicate with standard deviations. The regressive lines were made from the average of the triplicate values.
The mixtures ranging from 4/10 000 to 1/10 of the mutant plasmid DNA/the wild-type plasmid DNA were prepared. Since 1-10 ng of pMY189 plasmid DNA was recovered from $2.5 \times 10^5$ of H1299 cells transfected with 1 μg of the pMY189-8OHG plasmid in our previous studies (4,5), 100 pg and 1 ng of these mixtures were used as templates in QRT-PCR. QRT-PCR was done in triplicate, and the mean values of $\Delta Ct = -Ct (\text{sup}F-159T) - Ct (\text{pBR327-ori})$, see Materials and methods were plotted against the proportions of the mutant plasmid (Figure 1B). Linear correlations were obtained with coefficients of determination ($R^2$) of 0.983 and 0.985 for 100 pg and 1 ng of templates, respectively. The result indicated that G:C to T:A mutation frequencies at position 159 ranging from $10^{-3}$ to $10^{-5}$ could be evaluated by the QRT-PCR method using 100-$1000$ pg of the pMY189 plasmid.

Next, we assessed the mutation frequency of pMY189-8OHG plasmids, which were introduced into and replicated for 48 h in H1299 cells and H1299-OG3 cells, respectively, both by the QRT-PCR and conventional colony counting methods. H1299-OG3 is a H1299-derived stable transfectant of an OGG1-Ser326 cDNA expression vector, in which the OGG1-Ser326 protein was continuously expressed (5). G:C to T:A mutation frequencies at position 159 calculated by QRT-PCR in triplicate for H1299 cells and H1299-OG3 were $4.8 \times 10^{-2}$ (95% confidence interval (CI): 3.6-6.3 $\times 10^{-2}$) and 1.3 $\times 10^{-3}$ (95% CI: 0.8-2.1 $\times 10^{-3}$), respectively. Overall supF mutation frequencies were also estimated by the conventional colony plating method using an indicator-E.coli strain, KS40/ pKY241 (4,5). Mutation frequencies calculated based on the number of nalidixic acid-resistant colonies/number of all colonies were $3.1 \times 10^{-2}$ and $1.8 \times 10^{-3}$, respectively, therefore, G:C to T:A mutation frequencies at position 159 were deduced to be $3.0 \times 10^{-2}$ and $0.8 \times 10^{-3}$, respectively, based on the previous finding that 96.8 and 43.8% of all supF mutants from H1299 and H1299-OG3 cells carried G:C to T:A mutations at position 159 (5). In our previous study, mutation frequencies for a DNA sample estimated by the colony plating method deviated within 2-fold [2 SD for change ratios was 0.43 ($n = 22$ in ref. 5)] probably due to handling differences in the procedure of plating E.coli cells. Thus, G:C to T:A mutation frequencies were estimated by this method as being $3.0 \times 10^{-2}$ (95% CI: 1.7-4.3 $\times 10^{-2}$) and $0.8 \times 10^{-3}$ (95% CI: 0.5-1.1 $\times 10^{-3}$), respectively, indicating that frequencies obtained by the QRT-PCR and colony plating methods were consistent with each other. The QRT-PCR method was designed to specifically assess G to T mutations at the 8OHG-introduced site on the pMY189-8OHG plasmid, whereas the colony plating method was designed to assess frequencies of all mutations inactivating the supF gene, including mutations at sites other than the 8OHG-introduced site. Thus, we used the QRT-PCR method in the subsequent study, as this method was considered to enable us to assess frequencies of mutations induced by 8OHG on the pMY189-8OHG plasmid more accurately than the colony plating method.

Suppression of G:C to T:A mutation by exogenous OGG1 protein

We prepared adenoviruses, Ad-Ser326, Ad-Cys326 and Ad-Gln249, which transduct two polymorphic OGG1 proteins, OGG1-Ser326 and -Cys326, and an artificial mutant protein lacking 8OHG glycosylase activity in vitro, OGG1-Gln249 (18), respectively. We also prepared a control adenovirus, ΔE1, without an OGG1 cDNA insert. H1299 cells were infected with these adenoviruses at a MOI of 100. At 24, 48 and 72 h after infection, infected cells were harvested to assess exogenous OGG1 protein expression by western blot analysis using an anti-OGG1 antibody (Figure 2A). Exogenous OGG1 protein of 39 kDa in size was readily detectable in cells harvested at 24, 48 and 72 h after infection, and the difference in the amounts of each polymorphic OGG1 protein, which were calibrated based on the signal intensity of α-tubulin, was within 1.5 times. Thus, it was indicated that exogenous OGG1 proteins are stably expressed from 24 to 72 h after infection.

Next, to assess the suppressive ability of exogenous OGG1 proteins against G:C to T:A mutation, H1299 cells were infected again with Ad-Ser326, Ad-Cys326, Ad-Gln249 and ΔE1 viruses at a MOI of 100. Twenty-four hours after infection, half of the infected cells were harvested and subjected to western blot analysis to assess the amount of exogenous OGG1 proteins (Figure 2B, lower). The pMY189-8OHG plasmid was introduced into the remaining half of the cells and incubated for an additional 48 h. Then, the pMY189 plasmid was recovered from the transfected cells, and 8OHG-induced mutation frequencies were assessed by QRT-PCR (Figure 2B, upper). This plasmid was also introduced into uninfected H1299 cells and H1299-OG3 cells, respectively, as controls. Frequencies

Fig. 2. Adenoviral transduction of OGG1 proteins. (A) Exogenous OGG1 protein expression at 24, 48 and 72 h after infection assessed by western blot analysis. (B, upper) Mutation frequencies in the supF gene of pMY189-8OHG replicated in H1299 cells infected at a MOI 100 or not infected with adenoviruses. Data represent mean mutation frequencies estimated by QRT-PCR in triplicate with standard deviations. (B, lower) Exogenous OGG1 protein expression at 24 h after infection assessed by western blot analysis. OGG1 and α-tubulin proteins on the same blot were sequentially detected.
of G:C to T:A mutations at position 159 were at the $10^{-2}$ level in uninfected (MOCK) and ΔE1 or Ad-Gln249-infected cells, while they were reduced to at the $10^{-3}$ level in Ad-Ser326 and Ad-Cys326 infected and H1299-OG3 cells. We also assessed mutation frequencies of recovered plasmids by the conventional colony counting method, and these frequencies were similar to those estimated by QRT-PCR, supporting the accuracy of the QRT-PCR method in evaluating mutation frequency. More than 95% of G:C to T:A mutations at position 159 were found to be suppressed in Ad-Ser326 and Ad-Cys326 infected cells when a portion of recovered plasmids were transformed into an indicator E.coli and representative mutant plasmids were sequenced (data not shown). The expression levels of the exogenous OGG1 proteins were tens of times higher than those of endogenous OGG1 protein in lymphocytes and lung cancer cell lines (15; unpublished data). Thus, it was indicated that both the OGG1-Ser326 and -Cys326 proteins have the ability to suppress G:C to T:A mutations caused by 8OHG in human cells in vivo when large amounts of proteins were expressed. In contrast, it was also indicated that OGG1-Gln249 protein, an artificial mutant, lacks the ability to suppress G:C to T:A mutations caused by 8OHG in vivo.

Comparison of mutation suppressive ability between OGG1-Ser326 and -Cys326 in vivo

Next, to compare the mutation suppressive ability between OGG1-Ser326 and -Cys326 in a condition reflecting the physiological status, mutation frequencies of pMY189-8OHG were compared in H1299 cells infected by Ad-Ser326 and Ad-Cys326 viruses at lower MOIs (Figure 3A). We found that mutation frequencies in cells infected with ΔE1 and Ad-Gln249 viruses at a MOI of 100 were higher than uninfected cells (Figure 2B), although the differences were not statistically significant. Thus, considering the possibility that intracellular stress caused by adenoviral infection leads to the reduction of intracellular repair ability against 8OHG, infections in this comparative study were done at a MOI of 100 in total by compensating MOI to 100 with the ΔE1 virus to equalize intracellular stress caused by adenovirus infection among samples. Amounts of OGG1 protein in each sample were estimated accurately based on the signal intensity of exogenous OGG1 protein in the sample in comparison with that of purified GST-OGG1 protein on the same blot (Figure 3A). After standardizing the amount of protein loaded for each sample by the intensity of the α-tubulin signals, amounts of OGG1 protein in these samples were expressed as ng/μg of WCE calculated (Figure 3A). Amounts of exogenous OGG1 protein were changed in a MOI-dependent manner within a range of 0.08–0.46 ng/μg WCE. Amounts of endogenous OGG1 protein at a steady-state level in lymphocytes and lung cancer cell lines were estimated as being ~0.1 ng/μg WCE (15). Recent studies indicated that OGG1 expression levels are elevated several fold by intracellular stimuli, including oxidative stress (19–21). Thus, amounts of exogenous OGG1 protein transduced in cells were considered to be within or near the range where amounts of endogenous OGG1 protein physiologically vary.

Mutation frequencies of the pMY189-8OHG plasmid estimated by QRT-PCR were plotted against amounts of OGG1 protein in WCE (Figure 3B). Linear correlations were obtained between the mutation frequency in logarithm and the amount of OGG1 protein with coefficients of determination ($R^2$) of 0.922 and 0.945 for OGG1-Ser326 and -Cys326, respectively, indicating that the data fit well to the exponential equation (one-hit-model). The regressive lines for mutation frequencies ($y$) and amounts of OGG1 protein ($x$) were $y = 0.0399e^{-4.41x}$ for OGG1-Ser326 and $y = 0.0399e^{-2.64x}$ for OGG1-Cys326, respectively. The 95% CI of the slope for OGG1-Ser326 ($-5.05$ to $-3.77$) did not overlap that for OGG1-Cys326 ($-3.04$ to $-2.24$). The same experiment was performed independently. The regressive lines for mutation frequencies and amounts of OGG1 protein were $y = 0.0508e^{-3.06x}$ for OGG1-Ser326 and $y = 0.0508e^{-3.62x}$ for OGG1-Cys326, respectively (data not shown). The 95% CI of the slope for OGG1-Ser326
(−5.56 to −4.55) did not overlap that for OGG1-Cys326 (−4.00 to −3.24).

In both experiments, the slopes were significantly larger in OGG1-Cys326 transduced cells than in -Ser326 transduced cells (P < 0.05). The slopes account for efficiencies of mutation suppression (i.e. increase of mutation suppression per increase of OGG1 protein transduced). Thus, the result indicated that OGG1-Cys326 protein has a weaker suppressive ability than OGG1-Ser326 protein against G:C to T:A mutagenesis induced by 8OHG in human cells, and supported the previous results obtained by assays in E.coli cells and in vitro (9,14). The slopes estimated for OGG1-Ser326 and -Cys326 proteins, respectively, were different between the two experiments due to the difference in the transfection efficiency. The results of this comparative study were in agreement with those obtained by a complementation test in an E.coli (mutM mutY) strain that is defective in 8OHG repair (9) and by a test using OGG1 proteins, expressed in and purified from bacterial cells (14). As a result of the difference in the methods and conditions used for the comparison, we could not conclude whether differences in mutation suppressive ability observed in these studies were comparable with one another or not. However, the conditions used in this study were the most likely to have reflected the in vivo situation in human cells among the three studies, thus, the present results strengthened the idea that the OGG1-Cys326 allele confers cancer susceptibility due to its encoding the OGG1-Cys326 protein with a lower ability to prevent mutagenesis induced by 8OHG than OGG1-Ser326 protein.

In contrast to the present results, it was reported that 8OHG levels are significantly higher among lymphocytes irrespective of OGG1 genotypes (9,22). However, among patients undergoing chronic hemodialysis, 8OHG levels in lymphocyte DNA were significantly higher in homozygotes for the Cys326 allele than individuals with other OGG1 genotypes (22). The patients had higher levels of 8OHG in lymphocyte DNA than healthy individuals, probably due to reactive oxygen species produced by neutrophils activated during hemodialysis. Thus, it was speculated that intracellular activity of the polymorphic OGG1 proteins may be high enough to maintain 8OHG content at a steady level under conditions without severe oxidative stress and that the effects of differential repair activity between them may be manifested only under conditions with severe oxidative stress (22). Since the experimental conditions used in the present study were thought to have represented the condition with severe oxidative stress, i.e. intracellular amounts of 8OHG were artificially increased by transfection of pMY189-8OHG plasmid DNA transduced into each cell were different between the two experiments due to the difference in the transfection efficiency. The results of this comparative study were in agreement with those obtained by a complementation test in an E.coli (mutM mutY) strain that is defective in 8OHG repair (9) and by a test using OGG1 proteins, expressed in and purified from bacterial cells (14). As a result of the difference in the methods and conditions used for the comparison, we could not conclude whether differences in mutation suppressive ability observed in these studies were comparable with one another or not. However, the conditions used in this study were the most likely to have reflected the in vivo situation in human cells among the three studies, thus, the present results strengthened the idea that the OGG1-Cys326 allele confers cancer susceptibility due to its encoding the OGG1-Cys326 protein with a lower ability to prevent mutagenesis induced by 8OHG than OGG1-Ser326 protein.

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In this study, comparison was undertaken between cells in which either OGG1-Ser326 or -Cys326 protein was transduced, reproducing cells homozygous for the OGG1-Ser326 or -Cys326 alleles, respectively. However, cells reproducing heterozygotes for the polymorphism, i.e. cells in which equal amounts of OGG1-Ser326 and -Cys326 proteins were co-transduced, were not prepared in this study. This is because it was not possible to monitor the amounts of each OGG1 protein in such cells as antibodies against OGG1 that enable us to discriminate the two polymorphic OGG1 proteins are not available. Analysis of cells that reproduce heterozygotes will be worth investigating to confirm the differential ability between the two polymorphic OGG1 proteins. Interestingly, a recent report indicated that 8OHG glycosylase activity in homogenate from lymphocytes was similar irrespective of the OGG1 genotypes (23). Therefore, the difference in mutation suppressive ability in vivo between the two polymorphic OGG1 proteins might not simply reflect the difference in 8OHG glycosylase activity between them. Thus, further studies are needed to elucidate the activities/properties of OGG1 protein underlying the differential mutation suppressive ability between the two polymorphic OGG1 proteins.

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References


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