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

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OGG1 protein has an ability to suppress mutagenesis induced by 8-hydroxyguanine (8OHG), an oxidatively damaged promutagenic base. Here, the mutation suppressive ability was compared between two common polymorphic OGG1 proteins, OGG1-Ser326 and OGG1-Cys326, using a supF forward mutation assay employing an 8OHG-containing plasmid. Polymorphic OGG1 proteins were exogenously expressed by adenoviral transduction in H1299 human lung cancer cells, in which endogenous OGG1 protein was undetectable by western blot analysis. Mutations by 8OHG were more efficiently suppressed in OGG1-Ser326 transduced cells than OGG1-Cys326 transduced cells. The results indicated that OGG1-Cys326 has a lower ability to prevent mutagenesis by 8OHG than OGG1-Ser326 in vivo in human cells; supporting the results of recent association studies that OGG1-Cys326 is a risk allele for several types of human cancers.

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, from double-stranded DNA in vitro (2,3). We recently showed that the OGG1 protein suppresses G:C to T:A transversions caused by 8OHG in human cells in vivo (4,5). Ogg1 null mice showed higher contents of 8OHG and higher rates of G:C to T:A mutations in their DNA than wild-type mice, and were predisposed to lung adenocarcinoma and adenoma (6–8). These results indicate that the OGG1 gene plays a key role in preventing human carcinogenesis by suppressing G:C to T:A mutations by 8OHG.

A non-synonymous (associated with amino acid change) genetic polymorphism at codon 326, Ser326Cys, which we defined previously in the OGG1 gene (9), is a strong candidate as a genetic factor for cancer risk based on the following findings. Case-control (association) studies conducted by us and others on the polymorphism Ser326Cys, in the OGG1 gene, indicated that OGG1-Cys326 is a risk allele for a variety 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 endogeneous 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.
Preparation of adenoviruses to express polymorphic OGG1 proteins

Plasmid DNA with wild-type plasmid DNA) in duplicate to generate a standard 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 Ser326 cDNA in pcDNA3 was used for the template to produce cDNA (Stratagene, La Jolla, CA). A cDNA fragment covering the coding sequence (5'-CTGCGCAGAAA-3') and reverse (5'-AACCGGGGCCTTCCA-3') primers and 0.2 μM of TaqMan probe (5'-TET-ACCAATGAGATCCTACATCGGTCGACGA-TAMA-3') for the pBR327-ori amplicon. Both the supF and pBR327-ori amplicons included a DpnI restriction site, which can be used to exclude unreplicated plasmids retaining the bacterial methylation pattern. The amount of FAM and TET fluorescence liberated by the exonuclease degradation of the TaqMan probes during PCR amplification was measured using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) apparatus. PCR was run three or five times for each sample (see text). All samples in a comparative test were assayed on a single 96-well plate. Every PCR run included a seven-point standard (i.e. a serial dilution of mutant plasmid DNA with wild-type plasmid DNA) in duplicate to generate a standard curve. Ct (threshold cycle) values were determined for the test and reference loci for each sample three or five times and subtracted to obtain ΔCt [ΔCt = Ct (supF-159T) – Ct (pBR327-ori)]. The ΔCt values run three or five times were averaged, and the mutation frequency of each sample was estimated based on the averaged ΔCt values by referring to the standard curve.

Preparation of adeno viruses to express polymorphic OGG1 proteins

Full-length CDNs for the OGG1-Ser326 and -Cys326 type 1α transcripts were subcloned into the pcDNA3 plasmid (15; unpublished data). The OGG1-Ser326 CDN in pcDNA3 was used for the template to produce cDNA for OGG1-Ser326-249 using a Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). A CDN fragment covering the coding sequence (5’-CGGATTCGGAGAAGGAGCTCGGCGGACG-3’) and reverse (5’-AACCGGGGCCTTCCA-3’) primers and 0.2 μM of TaqMan probe (5’-TET-ACCAATGAGATCCTACATCGGTCGACG-3’) for the pBR327-ori amplicon. Both the supF and pBR327-ori amplicons included a DpnI restriction site, which can be used to exclude unreplicated plasmids retaining the bacterial methylation pattern. The amount of FAM and TET fluorescence liberated by the exonuclease degradation of the TaqMan probes during PCR amplification was measured using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA) apparatus. PCR was run three or five times for each sample (see text). All samples in a comparative test were assayed on a single 96-well plate. Every PCR run included a seven-point standard (i.e. a serial dilution of mutant plasmid DNA with wild-type plasmid DNA) in duplicate to generate a standard curve. Ct (threshold cycle) values were determined for the test and reference loci for each sample three or five times and subtracted to obtain ΔCt [ΔCt = Ct (supF-159T) – Ct (pBR327-ori)]. The ΔCt values run three or five times were averaged, and the mutation frequency of each sample was estimated based on the averaged ΔCt values by referring to the standard curve.

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 instead of G:C to T:A mutation frequencies at position 159. A SupF forward mutation assay

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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 solid filled 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. 100 pg: R² = 0.983, ΔCt = −1.76ln(X) – 1.83. 1 ng: R² = 0.985, ΔCt = −1.81ln(X) – 2.30.
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 × 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 ΔCt [ΔCt (supF-159T) - ΔCt (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^-2 to 10^-3 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 were deduced to be 3.0 × 10^-2 and 1.3 × 10^-3 (95% CI: 0.8-2.1 × 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 × 10^-2 and 1.8 × 10^-3, respectively, therefore, G:C to T:A mutation frequencies at position 159 were deduced to be 3.0 × 10^-2 and 0.8 × 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 × 10^-2 (95% CI: 1.7-4.3 × 10^-2) and 0.8 × 10^-3 (95% CI: 0.5-1.1 × 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: 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 transduce 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 \(\Delta E1\) 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 \(\Delta E1\) 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 \(\Delta E1\) 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 \(\alpha\)-tubulin signals, amounts of OGG1 protein in these samples were expressed as ng/\(\mu g\) 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/\(\mu g\) 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/\(\mu g\) 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 regression 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 regression 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
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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