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Expression of hOGG1 protein during differentiation of HL-60 cells

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Abbreviations: 8-oxo-G, 8-oxo-7,8-dihydroguanine; hOGG, human 8-oxo-G-DNA glycosylase; Vit. D_3, 1,25-dihydroxyvitmine D_3

Abstract

Human 8-oxo-G-DNA glycosylase 1 (hOGG1) is a DNA glycosylase to cleave 8-oxo-7,8-dihydroguanine (8-oxo-G), a mutagenic DNA adduct formed by oxidant stresses. Here, we examined hOGG1 protein expression and repair activity to nick a DNA strand at the site of 8-oxo-G during differentiation of hematopoietic cells using HL-60 cells. Overall expression of hOGG1 protein was increased during granulocytic differentiation of HL-60 cells induced by DMSO and monocytic differentiation by vitamine D_3. Greater level of hOGG1 protein was expressed in DMSO-treated cells. However, change in the DNA nicking activity was not in parallel with the change in hOGG1 protein expression, especially in PMA-treated cells. In PMA-treated cells, the level of hOGG1 protein was lowered, even though the DNA nicking activity was elevated, in a manner similar to the changes in serum-deprived HL-60 cells. These results indicate that hOGG1 expression change during differentiation of hematopoietic stem cells for adaptation to new environments. And the DNA cleaving activity may require additional factor(s) other than expressed hOGG1 protein, especially in apoptotic cell death.

Keywords: cell differentiation; DNA; glycosylation; reactive oxygen species; oxidative stress

Introduction

Reactive oxygen species (ROS) from endogenous metabolism or metabolism of xenobiotics are known to cause DNA damages. 8-oxo-7,8-dihydroguanine (8-oxo-G), a particularly abundant DNA adduct formed by ROS, is known to affect DNA synthesis and mRNA transcription. 8-oxo-G is highly mutagenic, yielding GC to TA transversion by mispairing with adenine upon DNA replication (Shibutani *et al.,* 1991; Michaels *et al.,* 1992). In addition, 8-oxo-G in DNA induced misincorporation of adenine instead of cytosine, resulting in lower promoter clearance during mRNA transcription (Viswanathan & Doetsch, 1998).

Most living organisms possess DNA glycosylases to remove 8-oxo-G from oxidatively damaged DNA. Like other organisms, human cells have two major types of 8-oxo-G DNA glycosylases, hOGG1 and hOGG2. While hOGG1 mainly acts on 8-oxo-G:C pair (Roldan-Arjona *et al.,* 1997), hOGG2 is suggested to backup hOGG1 by repairing 8-oxo-G:A pair, a poor substrate for hOGG1 that is formed when 8-oxoG is incorporated in the nascent strand from ROS-induced 8-oxo-dGTP during DNA replication (Hazra *et al.,* 1998). The both enzymes remove 8-oxoG and incise the resulting apurinic/apyrimidinic (AP) site by its accompanying AP-lyase activity through β-elimination mechanism.

Under several kinds of oxidative stresses, the DNA repair activity or the expression of OGG1 is reported to increase. Oxidative stress to the brain (Lin *et al.,* 2000) or the heart (You *et al.,* 2000) by ischemia-reperfusion caused increase in the DNA repair activity and/or the expression of OGG1 protein. And asbestos increased mRNA of hOGG1 together with 8-oxo-G in DNA in alveolar epithelial cells (Kim *et al.,* 2001). These data imply that induction of OGG1 may be one of the adaptive responses to oxidative stress.

In this study, we tried to evaluate physiological meaning of hOGG1 in inflammatory cells by determining change in hOGG1 protein expression and the DNA repair activity in differentiating HL-60 cells as a model. HL-60 cells are multipotent cells to be differentiated to either monocytic or granulocytic lineage (Collins *et al.,* 1979; Thompson *et al.,* 1988). To both directions, the differentiated cells have ability to generate ROS upon challenges by several stimuli. During differentiation of HL-60 cells, the activities or the expression of antioxidant enzymes such as Cu-Zn superoxide dismutase (Cu, Zn-SOD) (Auwerx *et al.,*

1989) and glutathione peroxidase (GPx) (Shen *et al.,* 1994) were changed. These changes may be considered as an adaptive phenomenon for differentiated cells to protect themselves from oxidant production upon stimulation or from increasing oxidant stress during differentiation (Covacci *et al.,* 2001).

Here, we report the changes in the DNA nicking activity at 8-oxo-G and the expression of hOGG1 protein during granulocytic or monocytic differentiation of HL-60 cells. In addition, we demonstrate their changes in serum-deprived HL-60 cells.

Materials and Methods

Materials

Oligonucleotide containing 8-oxo-G (21-mer: 5'-CAGCCAATCAGT**G^{oxo}**CACCATCC-3'; **G^{oxo}** was 8-oxo-G) was synthesized from Midland Certified Reagent Co. (Midland, TX). Oligonucleotide complementary to the 21-mer and 12-mer oligonucleotide were synthesized from Life Technologies (Rockville, MD). Enhanced ECL kits, MonoQ column and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech (Piscataway, NJ). Restriction enzymes, enterokinase, T4 polynucleotide kinase and cDNA synthesis kits were from Roche (Germany). Protease inhibitor cocktail, immunoblot membranes and protein assay kits were obtained from Biorad (Hercules, CA). Reagents for cell culture and 1,25-dihydroxy-vitamin D_3 (Vit. D_3) were obtained from Life Technologies (Rockville, MD) and Biomol (UK), respectively. All other reagents including PMA and DMSO were from Sigma (St. Louis, MO).

Cell culture

HL-60 cells were grown in RPMI media containing 10% fetal calf serum and 50 μM 2-mercaptoethanol. For monocytic differentiation, Vit. D_3 or PMA was added to culture media ($1.5\text{-}2\times10^6$ cells in a 60 mm dish in diameter) to a final concentration of 100 nM or 50 nM, respectively. To differentiate HL-60 cells to granulocytic lineage, DMSO was added to a final concentration of 1.25%. For serum deprivation, the cells were washed three times with serum-free RPMI and cultured in the same media.

Preparation of labeled of 21-mer duplex DNA containing 8-oxo-G

To determine the DNA nicking activity at 8-oxo-G, 21 mer oligonucleotide containing 8-oxo-G was 5' end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described previously (Roldan-Arjona *et al.,* 1997). The labeled oligonucleotide was annealed to its complementary oligonucleotide and used as a substrate to determine the DNA nicking activity. To confirm the cleavage site, 12-mer having the identical sequence 5' to 8-oxo-G and 3'-terminal hydroxyl group (3'-OH) was labeled at 5'-terminus.

Preparation of recombinant hOGG1 (rhOGG1)

6X His-tagged hOGG1 was expressed and purified as described previously with slight modification (Roldan-Arjona *et al.,* 1997). In brief, cDNAs were synthesized from total RNA of HeLa cells by reverse transcription, and the coding sequence from start to stop codon (1138 bps) was amplified by using mutant primers carrying *Bam*HI or *Sal*I restriction enzyme sites, respectively (forward, 5'-GTGGATCCATGCCTGCCCGCGCGCTTCT-3', *Bam*HI; reverse, 5'-GTGGTCGACCTAGCCTTCCGGCCCTTTG-3', *Sal*I). The amplified fragment was cloned to TA vector (Invitrogen, Carlsbad, CA), and then to pET-30a(+) (Novagen, Germany). Expression of rhOGG1 in transformed *E. coli* BL21 (DE3) was induced with 1 mM IPTG at 15^{o}C for 24 h. 6× His-tagged rhOGG1 in the *E. coli* lysate was purified with nickel nitriloacetic acid affinity column chromatography as manufacturer's instruction (Novagen). Excessive amino acids at the N-terminus of recombinant hOGG1 were removed by enterokinase and rhOGG1 was further purified by MonoQ column chromatography. The finally purified protein had 10 additional amino acids at N-terminus.

Preparation of cell extract for the DNA nicking assay

The cell extracts were prepared as described previously with slight modification (Chung *et al.,* 1991). At the indicated times, HL-60 cells were washed three times with cold PBS. The cells were ruptured in 200 μl of buffer A (50 mM Hepes-KOH, pH 8.0, 2 mM EDTA and 0.5 mM PMSF) by sonication on ice for 10 s. Streptomycin was added to a final concentration of 1% and the cell lysates were incubated on ice for 30 min. After centrifugation at 25,000 *g* for 20 min, the supernatants were extensively dialyzed against buffer B (buffer A+2 mM 2-mercaptoethanol and 50 mM KCl) and centrifuged at 25,000 *g* for 20 min. Protein concentrations of the supernatants were determined with bovine serum albumin as standards.

Assay of DNA nicking activity

The DNA nicking assay was done as described previously (Chung *et al.,* 1991). Reaction mixtures (100 μl) containing 0.4 pmol of labeled 21-mer duplex substrate DNA, 50 μg proteins of the cell extract or 2 μg of rhOGG1, 2% glycerol in buffer B were incubated at 37^{o}C for 1 h. Reactions were stopped by

adding 200 μl of tRNA solution (1 mg/ml tRNA, 0.45 M sodium acetate, pH 5.2) and 200 μl of phenol: chloroform (1:1, v/v). The mixtures were shaken vigorously and centrifuged at 20,000 *g* for 10 min. Nucleic acids in the supernatants were precipitated with cold ethanol and dried in a vacuum concentrator. Radioactivities were determined with a scintillation counter, and sequencing gel loading buffer was added to get the same radioactivity per unit volume (10-30 μl). The reaction products were separated in 20% denaturing (7 M urea) gel, and visualized on a phosphoimager (FLA-2000, Fuji, Japan).

Western analysis of hOGG1

At the indicated times, HL-60 cells were collected and washed with cold PBS. The collected cells were lysed in a buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.4% deoxycholate, 1% NP-40 and protease inhibitor cocktail) by sonication. After centrifugation and determination of protein concentrations in the supernatants, proteins (40 μg) were separated in 12% SDS-PAGE gels and transferred to PVDF membranes in a tank blotter. The membranes were blocked with 5% skim milk in T-TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20), and incubated with rabbit polyclonal anti-hOGG1 antibody (hOGG11-A; Alpha Diagnostic International, San Antonio, TX) at a titer of 1:2,000 in T-TBS containing 2.5% skim milk. The membranes were washed with T-TBS and incubated with anti-rabbit IgG antibody conjugated with horseradish peroxidase (Pierce, Rockford, IL) at a titer of 1:12,000. After washing, hOGG1 proteins in the membranes were detected with enhanced ECL kits (Amersham Pharmacia) and a chemiluminescence detector (LAS, Fuji, Japan).

Data analysis

The signals acquired from a phosphoimager or chemiluminescence detector were quantified by Image Gauge program (Fuji). Statistical significance of the quantified data from 4 to 6 samples in each group was determined by Student's t-test.

Results

Granulocytic differentiation

Granulocytic differentiation of hemopoietic cells were achieved by treating HL60 cells with 1.25% DMSO. At the indicated time points, the changes in the DNA cleaving activity at 8-oxo-G in the cell extracts were determined (Figure 1A).

The nicked fragment formed by the cell extracts (day 0, 2, 4, 6) locates at the same position of 12-mer having the identical sequence 5' to 8-oxo-G and 3'-OH, but, the cleavage fragments by hOGG1 are slightly upper to 12-mer (3'-AP). This difference might be caused by removal of 3'-AP site (3'-deoxyribophosphate) by other enzymes such as human apurinic/apyrimidinic endonuclease (HAP or APE/ref1) in the cell extracts after nicking of the affected strand by the accompanying AP lyase activity of hOGG1 through β-elimination (Boiteux and Radicella, 2000; Krokan *et al.,* 2000). The level of fragment at the position of 12-mer was measured as the DNA cleavage activity. Very little change in the DNA cleaving activity was observed at the 2nd day of DMSO treatment, and decreased at the 4th and the 6th day (Figure 1A). In contrast, the level of hOGG1 protein (about 38 kDa) increased gradually during the periods of DMSO treatment (Figure 1B). As indicated, the DNA cleavage activity was reduced to 80% or 50% of the control at the 4th or the 6th day, respectively (Figure 2). How-

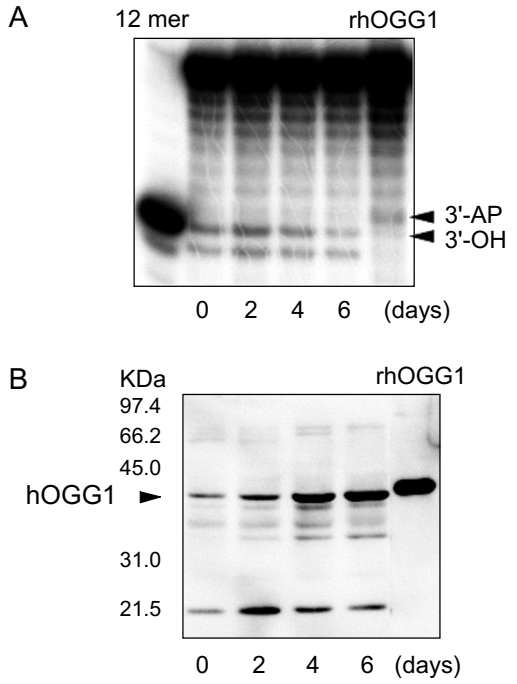

Figure 1. The DNA nicking activity and the expression of hOGG1 protein in HL-60 cells treated with 1.25% DMSO. At the indicated time points, HL-60 cells were harvested and the cell extracts were prepared. (A) The DNA nicking activity was determined by incubating substrate 21-mer duplex DNA containing 8-oxo-G at the 13rd position with the cell extracts. 3'-AP indicates a cleaved fragment by rhOGG1 which is 12-mer with 3'-terminal deoxyribophosphate. 3'-OH shows a fragment formed by cell extract, which locates at the same postion of 12-mer with 3'-terminal hydroxyl group (-OH). The intensity at 3'-AP is considered as the DNA nicking activity. (B) Immunoblot of hOGG1. 40 μg of total proteins were separated in 12% SDS-PAGE gels, and immunoblotting was done as described in Materials and Mothods. rhOGG1 shows recombinant hOGG1 (250 ng) having 10 additional amino acid residues.

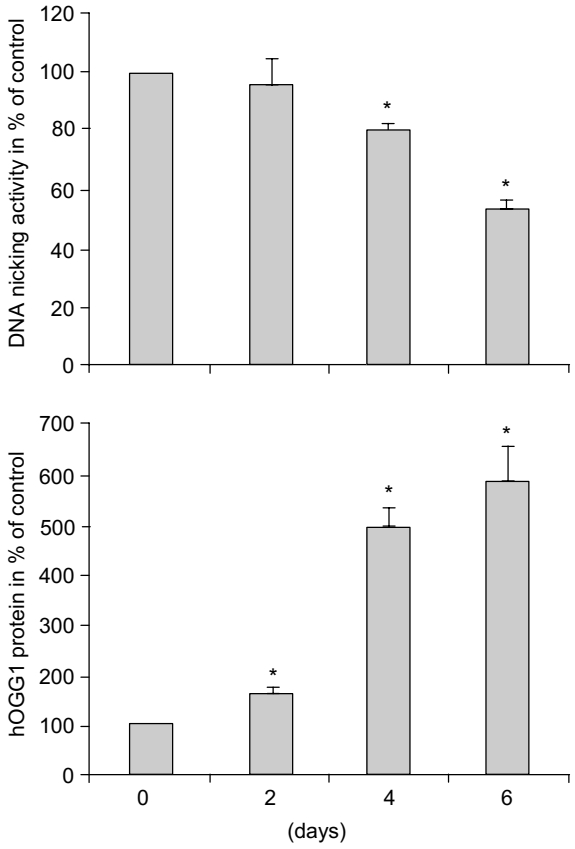

Figure 2. Quantitative analysis of the DNA nicking activity and the expression of hOGG1 protein in DMSO-treated HL-60 cells. (A) The DNA nicking activity. Image densities at 3'-AP in Figure 1A were analyzed with Image Guage program (Fuji), and were compared with a control group (day 0). (B) The amount of hOGG1 protein. Image densities in the immunoblots were quantified in the same manner, and were compared with a control group (day 0). Asterisks indicate statistical significance ($P < 0.05$) in comparison with a control group.

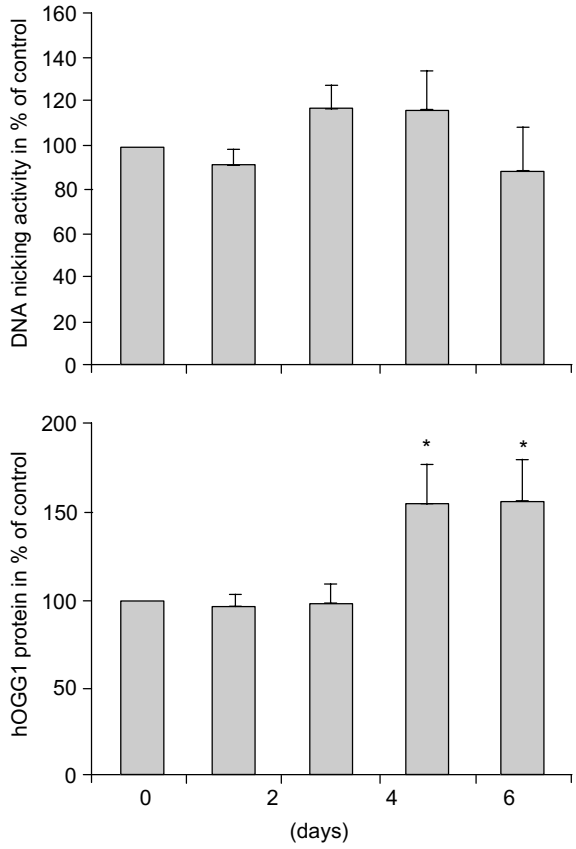

Figure 3. The DNA nicking activity and the expression of hOGG1 protein in HL-60 cells treated with 100 nM Vit. D_3. (A) The DNA nicking activity and (B) the amount of hOGG1 protein were determined and quantified as in Figure 1 and 2. Asterisks indicate statistical significance ($P < 0.05$) in comparison with a control group (day 0).

ever, the expression of hOGG1 protein significantly increased to 150%, 500% and 600% at the 2nd, the 4th and the 6th day of DMSO treatment, respectively.

Monocytic differentiation

There was hardly any change of the DNA cleavage activity was observed during the monocytic differentiation of HL-60 cells induced by Vit.D_3. However, the level of expressed hOGG1 protein was significantly increased at the 3rd and the 4th day of the treatment (Figure 3). In HL-60 cells treated with PMA, the DNA cleavage activity gradually elevated from 6 hrs after treatment, and reached to 250% level of the control at the 2nd day (Figure 4) while the hOGG1 protein expression was significantly reduced at the 1st and the 2nd day of PMA treatment.

Serum deprivation

PMA was known to induce apoptosis in HL-60 cells to a greater extent than other differentiation-inducing reagents (Ohta *et al.,* 1995), It is, however, not clear whether the discrepancy of the changes in between the DNA nicking activity and the expression of hOGG1 protein might be associated with accelerated apoptosis in PMA-treated HL-60 cells. To resolve such question, the DNA cleaving activity and the expression level of hOGG1 protein were compared using serum-deprived HL-60 cells (Figure 5). DNA cleaving activity and the expression level of hOGG1 protein were not changed at the 1st day of serum deprivation but discernable changes were observed at the 3rd day in both DNA cleaving activity and level of the protein expression. At the 3rd day of serum deprivation, the DNA cleaving activity was increased, but the expression level of hOGG1 protein was decreased, in a manner similar to the changes observed in PMA-treated HL-60 cells.

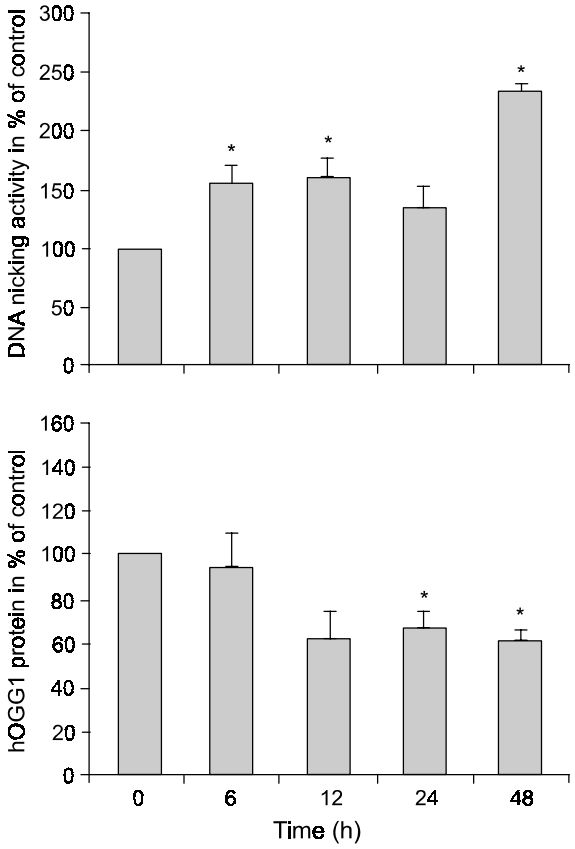

Figure 4. The DNA nicking activity and the expression of hOGG1 protein in HL-60 cells treated with 50 nM PMA. (A) The DNA nicking activity. (B) The amount of hOGG1 protein. Asterisks indicate statistical significance ($P < 0.05$) in comparison with a control group (day 0).

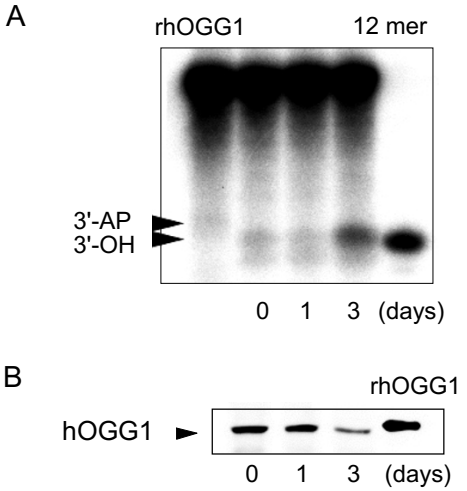

Figure 5. The DNA nicking activity and the expression of hOGG1 protein in serum-deprived HL-60 cells. After washing with serum-free media, HL-60 cells were cultured in serum-free media for the indicated periods. (A) The DNA nicking activity. (B) Immunoblot of hOGG1 protein.

Discussion

In this study, the expression of hOGG1 protein significantly increased in differentiating HL-60 cells, more remarkably by DMSO than by Vit. D_3. The differentiated HL-60 cells by DMSO have been considered as a model of human granulocytes (Collins *et al.,* 1979). In DMSO-treated HL-60 cells, PMA induced substantial production of ROS and caused increase of 8-oxo-G content in DNA (Takeuchi *et al.,* 1994). In addition, HL-60 cells are challenged by potentially harmful oxidant stress during differentiation (Covacci *et al.,* 2001). Therefore, the increased expression of hOGG1 protein may be considered as an adaptive response in differentiating HL-60 cells as well as in the mature granulocytes for keeping their genetic integrity and efficient mRNA transcription. However, we detected comparable changes in hOGG1 expression only in Vit. D_3-treated HL-60 cells, not in PMA-treated cells, even though both of the treated cells become capable to produce ROS when activated. Similar difference was reported in expression of Toll-like receptor 2 (Li *et al.,* 2002) and very low density lipoprotein (Kohno *et al.,* 1997) in HL-60 cells differentiated by PMA or Vit. D_3, even though both reagents induced expression of CD14, a differentiation marker for monocyte/macrophage lineage. The reason for this difference remains to be elucidated.

We observed the discrepancy of the changes in the DNA cleaving activity and the expression level of hOGG1 protein in DMSO-treated HL-60 cells. In granulocytic differentiation, the DNA cleaving activity decreased while the level of hOGG1 protein increased. In differentiated PC12 cell, the discrepancy in the OGG1 protein expression and the DNA cleaving activity was also reported (Stedeford *et al.,* 2001). A possible explanation for this discrepancy is that a certain kind of protein to bind DNA containing 8-oxo-G may be induced during the differentiation. Hazra *et al.* (1998) suggested the presence of a protein to inhibit the activity to cleave DNA containing 8-oxo-G in the whole cell extract of HeLa cells. However, the presence of the same activity and its change should be determined in differentiating HL-60 cell.

In monocytic differentiation by PMA treatment, the discrepancy was also observed but the changes were with the opposite direction; the DNA cleaving activity increased and the expression level of hOGG1 protein decreased. There may be several possibilities for this discrepancy. One is the induction of other 8-oxo-G DNA repair enzymes. Not only hOGG1 (Roldan-Arjona *et al.,* 1997) but also hOGG2 (Hazra *et al.,* 1998) remove 8-oxo-G and nick the DNA strand. In addition, *Drosophila* ribosomal S3 protein was also

known to have the DNA nicking activity (Yacoub *et al.,* 1996), even though a mammalian homologue has not been reported.

The second possibility for the discrepancy is the association with apoptosis. The expression of AP endonuclease (APE/ref-1) was down-regulated in HL-60 cells during monocytic differentiation (Robertson *et al.,* 1997). The authors proposed that the down-regulation may be associated with apoptosis as it became unnecessary to synthesis the new protein for base excision repair when the cells is committed to apoptosis. Judging from their suggestion, the decrease in the expression of hOGG1 observed in this experiment may be considered as an appropriate response of HL-60 cells committed to apoptosis. Moreover, as the AP site formed by hOGG1 was to be removed by APE (Ohta *et al.,* 1995; Krokan *et al.*, 2000), the decrease in the DNA cleaving activity would be expected. However, in this experiment, we observed the unexpected increase in the DNA cleaving activity in PMA-treated HL-60 cells.

Interestingly, these unexpected results were in agreement with the data from serum deprivation of HL-60 cells. Serum deprivation was known to induce apoptosis in HL-60 cells (Alexandre *et al.,* 2000). Serum deprivation caused the changes in the DNA cleaving activity and the expression of hOGG1 protein similar to those in PMA-treated HL-60 cells. Use of 2 mM EDTA in the DNA nicking assay, resulted in no cleavage of substrate DNA by Ca^{2+} or Mg^{2+}-dependent endonucleases activated in apoptosis. Even though no report has shown of 8-oxo-G directly mediating DNA cleavage in apoptosis (DNA laddering), the correlation of 8-oxo-G with apoptosis may be postulated from several experimental data. 8-oxo-dG induced apoptosis in KG-1 cells deficient of hOGG1 activity, even though the exact mechanism was not known (Hyun *et al.,* 2000). 8-oxo-G formed mainly at the internucleosomal sites by oxidative stress where DNA cleavage happens in apoptosis (Enright *et al.,* 1996), and 8-oxo-G augmented DNA strand breaks in bovine aortic endothelial cells treated by arsenite that induces apoptosis in the cells (Liu and Jan, 2000). In addition, significant increase was observed only in the content of 8-oxo-G among several kinds of oxidative base products in DNA when MOLT-4 cells was treated with doxorubicin at a concentration that did not induce oxidative stresses but apoptosis (Muller *et al.,* 1997). Even though our data from serum deprivation of HL-60 cells and the above reports may raise the possibility that 8-oxo-G mediate DNA cleavage in apoptosis, the postulation should be further confirmed by the experiment that whole cell extract deprived of OGG1 activity will still cleave the DNA containing 8-oxo-G and some endonucleases known to be activated in apoptosis will prove to cut the DNA containing 8-oxo-G.

Unique property of human OGCG1 and other BER pathways factors (enzymes) which could effect the DNA glycosylase activity has to be considered. Human OGG1 is distinct from other oxidized base-specific DNA glycosylases because of its extremely low turnover, weak AP lyase activity, and nonproductive affinity for the abasic (AP) site, its first reaction product. OGG1 is activated nearly 5-fold in the presence of AP-endonuclease (APE) as a result of its displacement by the latter (Hazra TK *et al.,* 2001)

It is generally accepted that DNA repair activities change during celluar differentiation (Nouspikel and Hanawalt, 2002). There are indications that DNA repair is attenuated at the global genome level (global genome repair, GGR), while the transcription-coupled repair (TCR) pathway could maintain genetic integrity of the transcribed stand in the active genes. In the point of cellular energy consumption, it may not become necessary to spend extra energy to repair inactive genes. In the case of 8-oxo-G, RNA polymerase II stalled at the lesion resulting in low promoter clearance, but sometimes bypassed it and inserted a cytosine residue or the incorrect adenine residue leading to a G:C to T:A transversion in RNA (Viswanathan and Doetsch, 1998; Kuraoka *et al.,* 2003). In this context, 8-oxo-G may be repaired in TCR pathway. However, there are conflicting results whether 8-oxo-G is repaired in TCR or GGR pathway (Le Page *et al.,* 2000a; Le Page *et al.,* 2000b; Sunesen *et al.,* 2002). Le Page *et al.* (2000a) suggested that OGG1 may not be involved in TCR and OGG1-independent pathway for the TCR of 8-oxo-G may exist *in vivo*.

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