Transcriptional mutagenesis mediated by 8-oxoG induces translational errors in mammalian cells

Da-Peng Dai[a,1], Wei Gan[a,1], Hiroshi Hayakawa[b,1], Jia-Lou Zhu[c,1], Xiu-Qing Zhang[c,1], Guo-Xin Hu[d], Tao Xu[d], Zhe-Li Jiang[d], Li-Qun Zhang[a], Xue-Da Hu[e], Ben Nie[d], Yue Zhou[f], Jin Li[a,g], Xiao-Yang Zhou[a], Jian Li[a], Tie-Mei Zhang[a], Qing He[h], Dong-Ge Liu[i], Hai-Bo Chen[j], Nan Yang[k], Ping-Ping Zuo[k], Zhi-Xin Zhang[l], Huan-Ming Yang[m], Yao Wang[a], Samuel H. Wilson[n], Yi-Xin Zeng[a], Jian-Ye Wang[a], Mutsuo Sekiguchi[b,2], and Jian-Ping Cai[a,2]

[a]MOH Key Laboratory of Geriatrics, Beijing Hospital, National Center of Gerontology, 100730 Beijing, People's Republic of China; [b]Frontier Research Center, Fukuoka Dental College, 8140193 Fukuoka, Japan; [c]Beijing Genomics Institute-Shenzhen, 518083 Shenzhen, People's Republic of China; [d]Department of Pharmacology, Wenzhou Medical College, Wenzhou, 325035 Zhejiang, People's Republic of China; [e]Cancer Institute and Hospital, Chinese Academy of Medical Sciences, 100021 Beijing, People's Republic of China; [f]Thermo Fisher Scientific Demonstration Laboratory, 201206 Shanghai, People's Republic of China; [g]Peking University Fifth School of Clinical Medicine, Beijing Hospital, 100730 Beijing, People's Republic of China; [h]Department of Cardiology, Beijing Hospital, 100730 Beijing, People's Republic of China; [i]Department of Pathology, Beijing Hospital, 100730 Beijing, People's Republic of China; [j]Department of Neurology, Beijing Hospital, 100730 Beijing, People's Republic of China; [k]Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, 100021 Beijing, People's Republic of China; [l]Beijing Red Cross Blood Center, 100088 Beijing, People's Republic of China; [m]James D. Watson Institute of Genome Sciences, 310058 Hangzhou, People's Republic of China; and [n]Genome Integrity and Structural Biology Laboratory, National Institutes of Health, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Edited by Philip C. Hanawalt, Stanford University, Stanford, CA, and approved March 16, 2018 (received for review November 9, 2017)

Reactive oxygen species formed within the mammalian cell can produce 8-oxo-7,8-dihydroguanine (8-oxoG) in mRNA, which can cause base mispairing during gene expression. Here we found that administration of 8-oxoGTP in MTH1-knockdown cells results in increased 8-oxoG content in mRNA. Under this condition, an amber mutation of the reporter luciferase is suppressed. Using second-generation sequencing techniques, we found that U-to-G changes at preassigned sites of the luciferase transcript increased when 8-oxoGTP was supplied. In addition, an increased level of 8-oxoG content in RNA induced the accumulation of aggregable amyloid β peptides in cells expressing amyloid precursor protein. Our findings indicate that 8-oxoG accumulation in mRNA can alter protein synthesis in mammalian cells. Further work is required to assess the significance of these findings under normal physiological conditions.

8-oxoG | MTH1 | RNA oxidization | amyloid β peptides

In aerobically growing cells, reactive oxygen and nitrogen species (ROS) are generated as byproducts of ATP production and oxygen utilization. Although most of these radicals are eliminated by the action of cellular antioxidant systems, some ROS still remain within the cell and damage various biologically important molecules, including proteins, lipids, and nucleic acids (1–3). Among the various types of oxidized purine and pyrimidine bases thus produced, 8-oxo-7,8-dihydroguanine (8-oxoG) appears to be the most important with respect to the maintenance and transfer of genetic information (4). Unlike other types of oxidized bases, 8-oxoG does not block nucleic acid synthesis but rather induces alternate base mispairing. When such mispairing occurs during DNA replication, base substitution mutations may result (5, 6). Likewise, the 8-oxoG mispairing that occurs during RNA synthesis may induce errors in gene expression (7).

Organisms are equipped with elaborate mechanisms for counteracting the deleterious effects of 8-oxoG (6, 8). One of these well-known mechanisms involves MTH1 enzymatic activity where deoxyribonucleoside triphosphate, 8-oxo-dGTP, is hydrolyzed to the monophosphate 8-oxo-dGMP, preventing 8-oxoG incorporation into DNA (9). The importance of MTH1 in the maintenance of DNA integrity was shown through a study of MTH1-deficient cells that revealed an increased frequency of spontaneous mutations compared with wild-type cells and that *MTH1*$^{-/-}$ mice developed more tumors than wild-type mice (3, 10). In addition to protecting DNA, MTH1 also plays a role in maintaining RNA integrity through its ability to degrade the 8-oxoG–containing ribonucleoside triphosphate, 8-oxoGTP. Thus, MTH1 can prevent an alteration in gene expression due to misincorporation of 8-oxoG into mRNA (9). Nevertheless, an influence of 8-oxoG in mRNA in mammalian cells is still unclear.

In the present study, we developed a luciferase reporter system for the detection of sequence-altered abnormal proteins, and we also applied a deep-sequencing method to measure ultra-rare mutations in oxidized mRNA to investigate the role of MTH1 in 8-oxoG–induced RNA mutagenesis in human cells. We found that the incorporation of 8-oxoG into the mRNA strand can induce translation errors, thus accelerating the production of the pathogenic amyloid β peptides in mammalian cells.

Results

Introduction of 8-oxoG into RNA of Living Cells. Since HeLa cells contain a relatively large amount of MTH1, we reduced the MTH1 content of cells using shRNA treatment. HeLa cells were

Significance

We had previously shown that 8-oxoG:A mismatch pairing can occur at both the DNA and RNA levels, thus causing base substitutions in DNA and translation errors, respectively. However, little attention has been paid to the biological significance of oxidized mRNA or to the clearance mechanism for such RNA lesions in mammalian cells. Here, we developed a sensitive reporter system to capture sequence-altered proteins that are translated from 8-oxoG–containing mRNAs. Also, we applied a deep-sequencing method to measure the content of these sequence-changed mRNAs to correlate the relationship between 8-oxoG–containing mRNAs and sequence-altered proteins. Additionally, we found that the accumulation of 8-oxoG in RNA can accelerate the production of pathogenic amyloid β peptides in mammalian cells.

Author contributions: M.S. and J.-P.C. designed research; D.-P.D., W.G., J.-L.Z., T.X., Z.-L.J., B.N., Y.Z., Jin Li, and N.Y. performed research; G.-X.H. contributed new reagents/analytic tools; D.-P.D., W.G., H.H., J.-L.Z., X.-Q.Z., T.X., Z.-L.J., L.-Q.Z., X.-D.H., B.N., Y.Z., Jin Li, X.-Y.Z., Jian Li, T.-M.Z., Q.H., D.-G.L., H.-B.C., N.Y., P.-P.Z., Z.-X.Z., H.-M.Y., Y.W., S.H.W., Y.-X.Z., J.-Y.W., and J.-P.C. analyzed data; and D.-P.D., W.G., H.H., J.-L.Z., S.H.W., M.S., and J.-P.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

[1]D.-P.D., W.G., H.H., J.-L.Z. and X.-Q.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: caijp61@vip.sina.com or mseki@college.fdcnet.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718363115/-/DCSupplemental.

Published online April 2, 2018.

Downloaded at Palestinian Territory, occupied on November 30, 2021

www.manaraa.com

infected with an shRNA retroviral vector containing a *MTH1*-targeting sequence and then selected for stable knockdown cells (Fig. 1*A*). To examine the biological effects of 8-oxoG in RNA, we introduced 8-oxoGTP into living cells by a hypotonic shift method (11). The addition of 8-oxoGTP to these MTH1-knockdown cells led to an increase in the 8-oxoG content in RNA (Fig. 1*B*). Two to four hours after the administration of 8-oxoGTP, the 8-oxoGsn/10^6 Gsn (guanosine) value in the MTH1-knockdown cells was twofold higher than in control cells. We noted that the RNA level of 8-oxoG detected in the 8-oxoGTP–treated cells decreased gradually to the control level within 12 h after the introduction of 8-oxoGTP. This decrease may be related to the preferential degradation of 8-oxoG–containing messenger RNA in HeLa cells (12).

Gene Expression Alteration via Insertion of 8-oxoG into Messenger RNA. Since 8-oxoG is able to pair with both adenine and cytosine (6, 8), the incorporation of 8-oxoG into messenger RNA can alter its coding properties. When an amber mutation was introduced into mRNA, we assumed that the °GAG codon (where °G represents an 8-oxoG–containing nucleotide) would pair with an anticodon sequence, e.g., 3′-CUC-5′ of tRNA, that codes for glutamate. Thus, an amount of sequence-altered protein carrying glutamate at this site will be produced within cells by bypassing the stop codon (Fig. 1*C*). Although we have evaluated this phenomenon in bacteria (7), the effect of 8-oxoG–containing mRNA in mammalian cells has remained unclear, because it is very hard to capture these sequence-altered proteins in living mammalian cells.

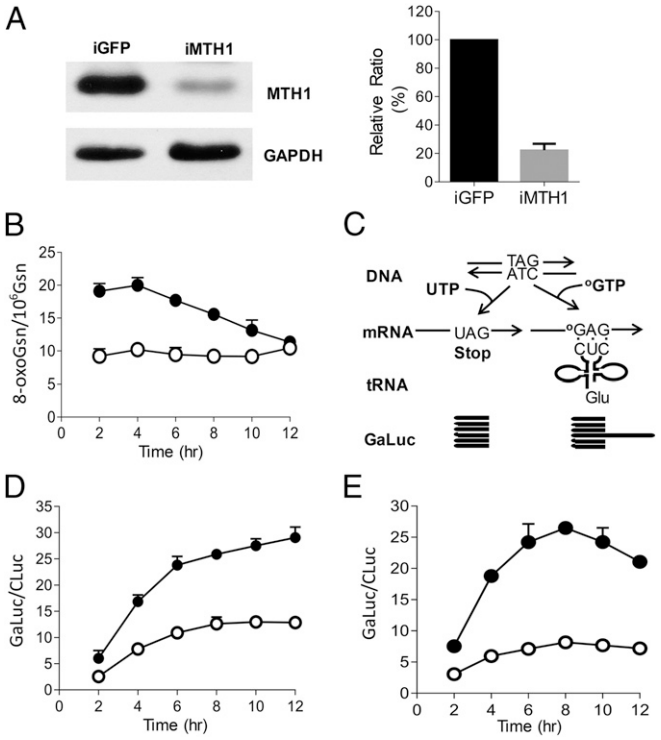

Fig. 1. Incorporation of 8-oxoG into RNA induced translation errors in HeLa cells. (*A*) Knockdown of the *MTH1* gene in HeLa cells. The amounts of MTH1 and GAPDH proteins in the *MTH1*-targeting (iMTH1) and *GFP*-targeting (iGFP) stable knockdown cells were measured using a Western blot analysis. The relative ratios of the MTH1 expression are presented ($n = 4$). (*B*) The 8-oxoG content in cellular RNA of the MTH1-knockdown cells. The cells were treated with 1.8 mM 8-oxoGTP, and the amount of 8-oxoG in the RNA was measured ($n = 3$) (•). As a control, cells were treated with KHB buffer (*SI Materials and Methods*) alone (o). (*C*) A schematic representation of the abnormal protein synthesis induced by 8-oxoG incorporation into messenger RNA. °G, 8-oxoG. (*D*) The GaLuc/CLuc values in MTH1-knockdown (•) and control iGFP (o) cells. Twenty-four hours after transfection with pIRES-GCluc, the cells were treated with KHB buffer containing 1.8 mM 8-oxoGTP. At the indicated times, the culture medium was retrieved, and the two types of luciferase activities were measured, as described in *SI Materials and Methods* ($n = 5$). (*E*) The GaLuc/CLuc values in the MTH1-knockdown cells transfected with an empty vector (•) and those transfected with *MTH1* cDNA to overproduce MTH1 proteins (o).

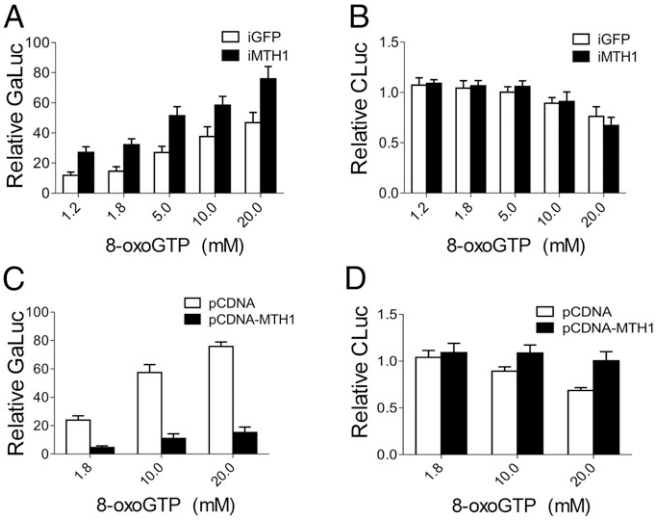

Fig. 2. Expression of GaLuc and CLuc in response to 8-oxoG. (*A*) The relative GaLuc signals in the MTH1-knockdown (iMTH1, closed bars) and control (iGFP, open bars) cells upon the application of 8-oxoGTP. Twenty-four hours after transfection with the reporter, the cells were permeabilized in the presence of various concentrations of 8-oxoGTP and then were cultured in medium for 12 h. The relative GaLuc signal was calculated by dividing the value for the 8-oxoGTP–treated cells by that for the nontreated control cells. (*B*) The relative CLuc signals in the MTH1-knockdown and control cells. The CLuc signal was calculated in a similar manner to the GaLuc signal. (*C*) The relative GaLuc signals in the MTH1-knockdown cells transfected with an empty vector (open bar) and those transfected with *MTH1* cDNA to overproduce MTH1 proteins (closed bar). The experimental conditions were as described in *A*. (*D*) The relative CLuc signals in the MTH1-knockdown cells transfected with an empty vector (open bar) and those transfected with *MTH1* cDNA to overproduce MTH1 protein (closed bar).

To quantify the extent of the production of this type of altered protein in mammalian cells, we developed a system using *Gaussia princeps* luciferase (GaLuc) (13) as the reporter. The luciferase is excreted outside the cell and can be easily assayed in the medium. When 8-oxoGTP was externally supplied, certain U bases were replaced with 8-oxoG, resulting in the formation of active GaLuc enzyme. As a reference, the sequence for *Cypridina noctiluca* luciferase (CLuc) was also included in the vector (Fig. S1) and was coexpressed. Since the activities of GaLuc and CLuc can be easily assayed with different reaction buffers, the GaLuc/CLuc ratio indicates the level of amber suppression, and we measured this ratio throughout the time course of the experiment (Fig. 1*D*). The MTH1-knockdown cells exhibited a higher level of suppression against the amber codon in response to 8-oxoG addition, whereas the control GFP-knockdown cells had only a weak response. Conversely, overproduction of MTH1 greatly reduced the GaLuc/CLuc ratio in the cells (Fig. 1*E*). Application of higher concentrations of 8-oxoGTP increased the level of GaLuc expression (Fig. 2*A*), while CLuc expression was not significantly affected and was inhibited at high concentrations of 8-oxoGTP (Fig. 2*B*). Overproduction of MTH1 resulted in a reversal of the effects of 8-oxoGTP on GaLuc and CLuc production (Fig. 2 *C* and *D*). These findings indicate that 8-oxoG introduction into RNA can induce alterations in gene expression.

GENETICS

Downloaded at Palestinian Territory, occupied on November 30, 2021
www.manaraa.com

RNA Mutagenesis Induced by 8-oxoG. To correlate changes in the GaLuc signal with RNA nucleotide alterations, we amplified the RNA sequences via RT-PCR and determined the frequency of base substitutions using second-generation sequencing technology. Fig. S2 shows the workflow of this procedure, which consisted of two different PCR amplification steps. In the first step, the assignment of a unique identifier (UID) was made at each end of a PCR fragment using a pair of 5′-tailed primers that contained nine degenerate N bases (creating UIDs for each amplicon). The second PCR amplification step used universal primers containing the sequences required for attachment to the Hiseq2000 sequencer, in which each uniquely tagged template was amplified to obtain a large number of daughter molecules with the same double-UID sequences. This allowed the elimination of consequent replication or sequencing errors in the subsequent data analysis.

We then evaluated the accuracy of this method by mixing GaLuc mRNA with a small amount of mutated GaLuc mRNA, which contained three independent base changes at the positions shown in Fig. 3*A*. The RNA mixture was subjected to the RT-PCR amplification procedure, and the resulting sequences were determined. Briefly, mutated GaLuc RNA was diluted in a stepwise fashion to concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} with wild-type GaLuc RNA, and the RNA mixture was then used as the template for RT-PCR amplification. As shown in Fig. 3*B* and in Table S1, this method allowed the detection of very rare mutant RNA among a large number of wild-type RNA molecules, with a detection threshold as low as 10^{-5}.

Using this method, we investigated the level of 8-oxoG–induced mRNA mutagenesis in living cells (Fig. 3*C*). When 8-oxoGTP was introduced into HeLa cells, the frequency of a U (T for cDNA)-to-G change at positions 304 and 323 increased strongly, and we observed a substantially larger effect of 8-oxoGTP in the MTH1-knockdown cells compared with the MTH1-overexpressing cells. At the other site, 307, administration of 8-oxoGTP resulted in a similar but much lower effect. The quantitative differences observed at the three sites may be due to differences in the surrounding sequences. For site 304, the base substitution changed an amber nonsense codon, UAG, to a sense codon encoding for glutamate; such a change could reduce mRNA nonsense-mediated decay (14). The results in this study are complex, but overall they indicate that human MTH1 counteracts oxidative stress by maintaining the fidelity of gene expression.

Amyloid Peptide Secretion Is Increased in 8-oxoGTP–Treated CHO_APP_iMTH1 Cells. Previous studies had shown that brain amyloid is composed almost entirely of the 4-kDa aggregable hydrophobic amyloid β (Aβ) peptides, such as Aβ1–40 or Aβ1–42 (15). Two amino acid substitutions (Lys to Asn at residue 595 and Met to Leu at position 596) in the amyloid precursor protein (APP) can cause higher β secretase-cleaving activity, resulting in a six- to eightfold increment of Aβ versus normal β-APP (16). To illustrate the utility of the findings described above, we hypothesized that accumulation of 8-oxoG in APP mRNA would lead to sequence changes in APP proteins, subsequently causing alterations in the sites where secretase cleaves. In this case, an increase in the amount of Aβ peptides would be observed in the brains of Alzheimer's disease (AD) patients compared with that in normal subjects. To investigate whether the accumulation of 8-oxoG in RNA can influence the production of Aβ protein in mammalian cells, we developed CHO_APP_iMTH1 cells, a stable CHO cell line with low expression of MTH1 and high expression of human APP (Fig. S3), and fed them with 8-oxoGTP in the culture medium. We found that the basal 8-oxoGsn/10^6Gsn ratio in untreated cells was 6.5, but it increased to 25.1 in treated cells after the application of 8-oxoGTP (Fig. 4*A*).

Next, we determined the overall amount of three typical Aβ peptides (Aβ1–x, Aβ1–40, and Aβ1–42) in the culture medium using an ELISA kit. After normalizing by the cell number, the 8-oxoGTP–treated cells showed a 1.6-fold, 1.8-fold, and 1.7d-fold increase in the levels of the Aβ1–x, Aβ1–40, and Aβ1–42 peptides, respectively, relative to untreated control. This demonstrated that the accumulation of 8-oxoG in RNA significantly increased Aβ secretion in vivo (Fig. 4 *B*–*D*).

To better understand the detailed characteristics of secreted Aβ peptides, we performed a nanoscale LC (nLC)-orbitrap MS analysis on peptides after immunoprecipitation by Aβ-specific monoclonal antibodies. We identified 28 different types of Aβ products specific to the treated samples (Fig. 4*E*). There were 22 Aβ products present in both control and treated samples; however, 14 of these products were up-regulated in the 8-oxoGTP–treated samples (ratio >2) under acidic separation conditions (Fig. 4*F*). To quantify the Aβ products that were of relatively long length, such as Aβ1–40 and Aβ1–42, we performed another

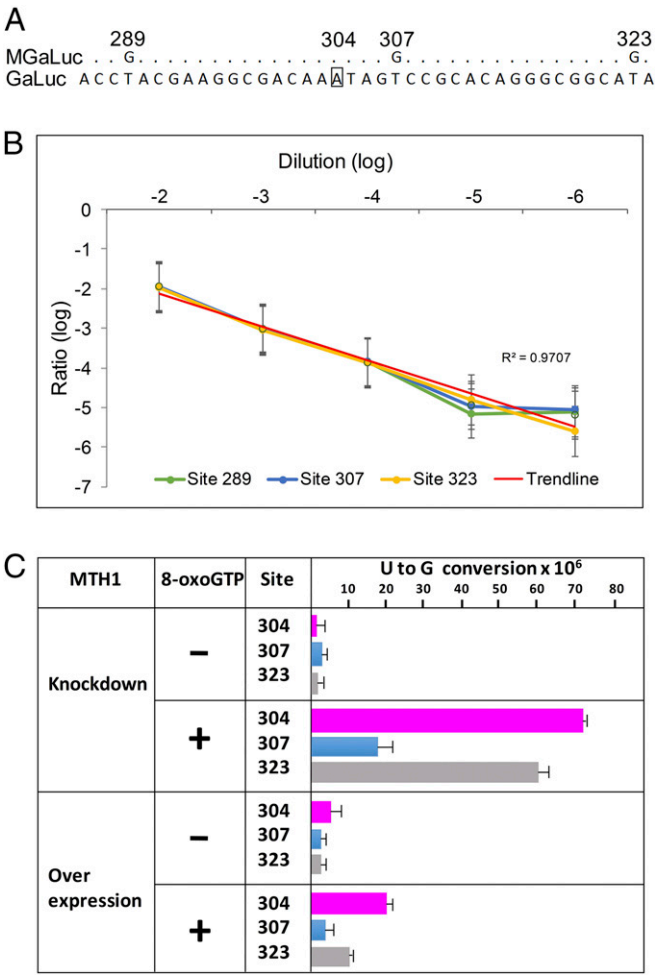

Fig. 3. Evaluation of the mutation-detecting system and 8-oxoG–related transcriptional mutagenesis. (*A*) The target sequences with preassigned mutations. The numbers above the sequences indicate the position of the T nucleotides included in the target regions of the cDNA in wild-type GaLuc and site-mutated GaLuc (MGaLuc) sequences. MGaLuc mRNA standard includes three predesigned T(U)→G mutation sites (289, 307, and 323) around the internal control site (304). (*B*) Graphic presentation of evaluation data of the mutation-detecting system. The *y* axis represents the log ratio of the concentration of T(U)→G mutations detected in this experiment. The red line represents the fitted linear curve with $R^2 = 0.97$. Wild-type GaLuc mRNA was mixed with mutated GaLuc mRNA standards via gradient dilution at the following series of concentrations: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. (*C*) Three T sites within the GaLuc target sequence (sites 304, 307, and 323) were scored to estimate the rate of U-to-G conversion under various conditions. Mutations were detected using the second-generation sequencing platform.

Downloaded at Palestinian Territory, occupied on November 30, 2021
www.manaraa.com

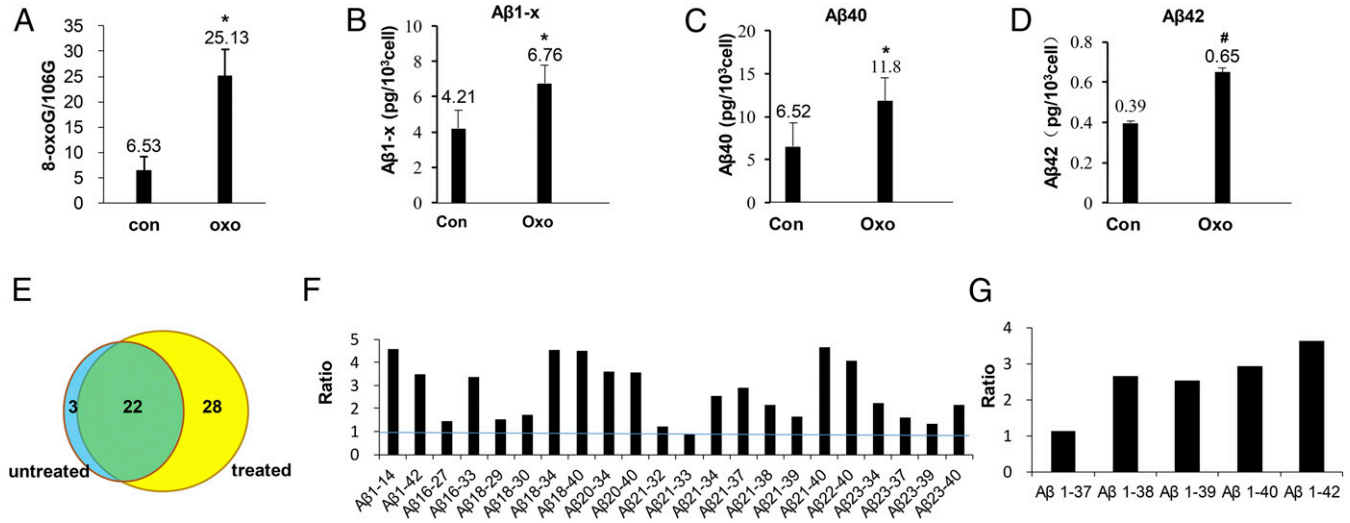

Fig. 4. Changes in the Aβ peptide expression pattern in 8-oxoGTP–treated CHO_APP_iMTH1 cells. (*A*) The effect of externally supplied 8-oxoGTP on the 8-oxoG content of the RNA of CHO_APP_iMTH1 cells. 8-oxoGTP was supplied to cells by osmotic pressure; then the cells were cocultured with 1 mM 8-oxoGTP for an additional 24 h to increase the efficiency of incorporation. The 8-oxoG content of the RNA was calculated as 8-oxoGsn/10^6Gsn ($n = 3$). Significance was determined using the Student's *t* test: $^*P < 0.05$. (*B–D*) RNA oxidation enhances the secretion of typical Aβ peptides in vitro. The amounts of Aβ1–x, Aβ1–40, and Aβ1–42 in the culture medium of 8-oxoGTP–treated CHO_APP_iMTH1 cells were quantified using a commercialized ELISA kit. The results were normalized to the cell number ($n = 3$), and significance was determined using the Student's *t* test: $^*P < 0.05$, $^{\#}P < 0.01$. (*E*) The overall types of Aβ products identified in the control and 8-oxoGTP–treated samples using nLC-orbitrap MS in an acid mobile phase. (*F*) The relative quantification of 22 Aβ products identified in both treated and untreated samples using nLC-orbitrap MS in an acid mobile phase. For each pair, the ratio of the treated/untreated samples is presented. (*G*) Quantification of large Aβ fragments in the medium of 8-oxoGTP–treated and untreated CHO_APP_iMTH1 cells using nLC-orbitrap MS in an alkaline mobile phase.

MS analysis on an UltiMate 3000 system (Thermo Fisher Scientific) and found that Aβ1–38, 1–39, 1–40, and 1–42 were up-regulated (ratio >2), while Aβ1–37 was only slightly different ($0.5 <$ ratio < 2) in the 8-oxoGTP–treated samples (Fig. 4*G* and Fig. S4). Overall, these data suggest that an increase in 8-oxoG content in mRNA could lead to an accumulation of Aβ protein in mammalian cells.

Discussion

The 8-oxoGTP in the cellular nucleotide pool can be used in RNA synthesis, as RNA polymerase II utilizes 8-oxoGTP as one of its substrates (17). On the other hand, 8-oxoGTP cannot be used for DNA synthesis, since ribonucleoside diphosphate reductase, which converts GDP and other ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates, is unable to reduce 8-oxoG–containing ribonucleotides (17, 18). It appears, therefore, that externally supplied 8-oxoGTP may be used for RNA synthesis. We have taken advantage of this specificity for RNA and have investigated the effects of 8-oxoG on gene expression.

Taddei et al. (7) used the *lacZ* amber system to investigate the specific role of the *Escherichia coli* MutT enzyme in preventing transcriptional errors caused by 8-oxoG. However, this system cannot be used directly in mammalian cells, because the amounts of oxidized mRNA-induced sequence-altered abnormal proteins are so tiny that another, more sensitive and stable reporter enzyme must to be included in the analysis. In this study, we developed a method using GaLuc as the reporter. This enzyme, which consists of only 185 amino acid residues, is accumulatively excreted into the culture medium and remains stable (13, 19). When 8-oxoG is introduced into sites of the amber codon in the messenger RNA for GaLuc, it is expected that a small amount of active enzyme will be produced (Fig. 1*C*). By introducing the sequence for CLuc into the reporter construct, which allowed us to estimate the extent of suppression more precisely, it was possible to demonstrate that MTH1-knockdown cells exhibit a high level of suppression of amber mutations in response to externally supplied 8-oxoGTP. Thus, we could correlate the 8-oxoG content in RNA with the biological response.

To better understand the effects of 8-oxoG on translational errors under normal physiological conditions, we also examined wild-type cells treated with H_2O_2. Under this oxidative stress condition, about 20% up-regulation in the GaLuc signal could be detected at 2–8 h after treatment. In contrast, a 13-fold higher amber suppression over control was observed in 8-oxoGTP–treated wild-type cells (Fig. 1*D*). We believe that, in addition to the oxidation of GTP, H_2O_2 can cause oxidation of dNTPs and damage DNA stands, proteins, and lipids. Such damage could potentially obscure the effect of 8-oxoG–related amber suppression in our system. Further work will be required to assess the effects of 8-oxoG on translation more thoroughly.

To detect mutations arising in the RNA transcripts, we developed a sequencing method based on a second-generation sequencing platform, which enabled us to detect as few as 10^{-5} mutations in the transcripts (Fig. 3*B*). When a large amount of 8-oxoGTP was introduced into the cells depleted of MTH1 activity by gene knockdown, the frequency of U-to-G changes in mRNA transcripts (T-to-G in cDNA) increased significantly. Thus, the GaLuc signal in our system corresponds well to the change of 8-oxoG content in mRNA.

Nunomura et al. (20) suggested that RNA oxidation is a prominent feature of neuronal vulnerability in patients with AD. Here, we clarified the causal relationship between RNA oxidation and Aβ plaque formation using CHO cells with 8-oxoGTP. Our amino acid sequence analysis revealed that 8-oxoGTP–treated cells secreted 28 previously unobserved Aβ peptides, in addition to a greater amount of pathogenic Aβ peptides (e.g., Aβ1–42) (Fig. 4). These results suggest that the existence of a large amount of 8-oxoG in the RNA could promote the secretion of pathogenic Aβ peptides in vivo.

How does RNA oxidation affect Aβ production and metabolism? Previous studies have shown that RNA oxidation is not a random event but occurs rather selectively, and some RNA species closely related to AD are more susceptible to oxidative

Downloaded at Palestinian Territory, occupied on November 30, 2021
www.manaraa.com

damage (21). As we demonstrated with our reporter system, 8-oxoG–containing mRNA may be translated to abnormal protein. It is reasonable to assume that oxidized RNA induces the formation of altered forms of APPs, making these proteins more vulnerable to degradation into pathogenic Aβ variants. Recently, Szaruga et al. (22) reported that some pathogenic substitutions (such as T43I, I45F, and V46I) located around the γ-cleavage sites can destabilize the γ-secretase–Aβn complexes and consequently enhance the product dissociation and the release of long, amyloidogenic Aβ. We suspect that some mutations caused by the 8-oxoG–containing mRNA maybe have similar effects on the stability of enzyme–substrate complex and result in the acceleration of longer, amyloidogenic Aβ productions. Alternatively, RNA oxidation may result in a decreased level of enzymatic activity so that the degradation of toxic Aβ peptides is reduced or prevented. All these mechanisms would contribute to the accumulation of plaque, as observed in the brains of AD patients.

A previous study revealed that 8-oxoG can be regarded as a representative marker of oxidative RNA lesions (23). An increased amount of 8-oxoG has been noted in RNA in the hippocampi of senescence-accelerated SAMP8 mice, which was correlated with a decreased expression level of MTH1 in these mice. Notably, these mice exhibited early aging and declining abilities for both learning and memory compared with age-matched control mice (24). In several previous studies, we revealed that both the 8-oxoG content in the brain RNA and the amount of 8-oxoguanosine in urine increase more rapidly in elderly mice, rats, and monkeys (24–27). These results are consistent with our findings here that indicate that 8-oxoG–mediated RNA dysfunction is a causative factor of aging and accelerates the development of age-related disorders.

Overall, our work here shows that 8-oxoG in mRNA can lead to translation errors in protein of mammalian cells; the accumulation of such sequence-altered proteins could have important implications in mammalian cells.

Materials and Methods

Detailed information on methods, including the construction of stable cell lines, 8-oxoGTP treatment, development and detection of the reporter system, second-generation sequencing of transcribed messenger RNAs, measurement of 8-oxoG content in RNA by HPLC-MS/MS, and detection of Aβ peptides by nLC-orbitrap MS are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Ke-Tong Wang of Liuhetong Corporation for technical support and services and the members of the Institute of Geriatrics of the Ministry of Health for their assistance and advice. This work was supported by the Key International Science and Technology Cooperation Project of China Grant 2006DFB31410, National Natural Science Foundation of China Grants 81171028 and 81571058, and NIH/National Institute of Environmental Health Sciences Intramural Research Program Project Z01 ES050159.

1. Fridovich I (1978) The biology of oxygen radicals. *Science* 201:875–880.
2. Demple B, Linn S (1980) DNA N-glycosylases and UV repair. *Nature* 287:203–208.
3. Sekiguchi M, Tsuzuki T (2002) Oxidative nucleotide damage: Consequences and prevention. *Oncogene* 21:8895–8904.
4. Kasai H, Nishimura S (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res* 12:2137–2145.
5. Wood ML, Dizdaroglu M, Gajewski E, Essigmann JM (1990) Mechanistic studies of ionizing radiation and oxidative mutagenesis: Genetic effects of a single 8-hydroxyguanine (7-hydro-8-oxoguanine) residue inserted at a unique site in a viral genome. *Biochemistry* 29:7024–7032.
6. Michaels ML, Cruz C, Grollman AP, Miller JH (1992) Evidence that MutY and MutM combine to prevent mutations by an oxidatively damaged form of guanine in DNA. *Proc Natl Acad Sci USA* 89:7022–7025.
7. Taddei F, et al. (1997) Counteraction by MutT protein of transcriptional errors caused by oxidative damage. *Science* 278:128–130.
8. Maki H, Sekiguchi M (1992) MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* 355:273–275.
9. Takagi Y, et al. (2012) Human MTH3 (NUDT18) protein hydrolyzes oxidized forms of guanosine and deoxyguanosine diphosphates: Comparison with MTH1 and MTH2. *J Biol Chem* 287:21541–21549.
10. Tsuzuki T, et al. (2001) Spontaneous tumorigenesis in mice defective in the MTH1 gene encoding 8-oxo-dGTPase. *Proc Natl Acad Sci USA* 98:11456–11461.
11. Koberna K, et al. (1999) Nuclear organization studied with the help of a hypotonic shift: Its use permits hydrophilic molecules to enter into living cells. *Chromosoma* 108: 325–335.
12. Ishii T, Hayakawa H, Sekiguchi T, Adachi N, Sekiguchi M (2015) Role of Auf1 in elimination of oxidatively damaged messenger RNA in human cells. *Free Radic Biol Med* 79:109–116.
13. Wurdinger T, et al. (2008) A secreted luciferase for ex vivo monitoring of in vivo processes. *Nat Methods* 5:171–173.
14. Lewis BP, Green RE, Brenner SE (2003) Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc Natl Acad Sci USA* 100:189–192.
15. Gandy S (2005) The role of cerebral amyloid beta accumulation in common forms of Alzheimer disease. *J Clin Invest* 115:1121–1129.
16. Citron M, et al. (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 360:672–674.
17. Hayakawa H, et al. (1999) Metabolic fate of oxidized guanine ribonucleotides in mammalian cells. *Biochemistry* 38:3610–3614.
18. Elledge SJ, Zhou Z, Allen JB (1992) Ribonucleotide reductase: Regulation, regulation, regulation. *Trends Biochem Sci* 17:119–123.
19. Verhaegent M, Christopoulos TK (2002) Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization. *Anal Chem* 74:4378–4385.
20. Nunomura A, et al. (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci* 19:1959–1964.
21. Shan X, Tashiro H, Lin CL (2003) The identification and characterization of oxidized RNAs in Alzheimer's disease. *J Neurosci* 23:4913–4921.
22. Szaruga M, et al. (2017) Alzheimer's-causing mutations shift Aβ length by destabilizing γ-secretase-Aβn interactions. *Cell* 170:443–456.e14.
23. Zhu X, et al. (2004) Oxidative stress signalling in Alzheimer's disease. *Brain Res* 1000: 32–39.
24. Song XN, et al. (2011) Oxidative damage to RNA and expression patterns of MTH1 in the hippocampi of senescence-accelerated SAMP8 mice and Alzheimer's disease patients. *Neurochem Res* 36:1558–1565.
25. Gan W, et al. (2012) Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescence-accelerated mice analyzed by LC-MS/MS: Urinary 8-oxoguanosine as a novel biomarker of aging. *Free Radic Biol Med* 52: 1700–1707.
26. Shi F, et al. (2012) Oxidative damage of DNA, RNA and their metabolites in leukocytes, plasma and urine of Macaca mulatta: 8-oxoguanosine in urine is a useful marker for aging. *Free Radic Res* 46:1093–1098.
27. Nie B, et al. (2013) Age-dependent accumulation of 8-oxoguanine in the DNA and RNA in various rat tissues. *Oxid Med Cell Longev* 2013:303181.

Downloaded at Palestinian Territory, occupied on November 30, 2021
www.manaraa.com