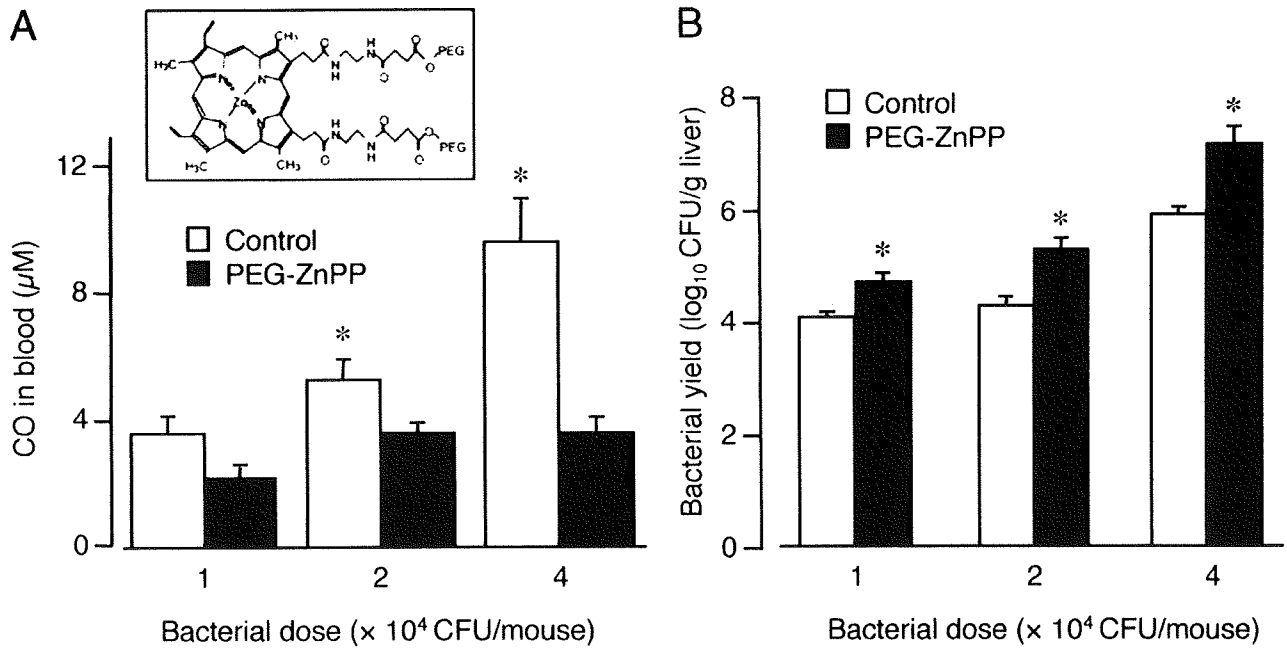

FIGURE 5. Effects of HO inhibition by PEG-ZnPP on growth of bacteria in mouse liver. *A*, Wild-type mice were infected with *S. typhimurium* at different doses and were treated or not treated with PEG-ZnPP. CO production in blood was measured at day 5 after infection. Data are means ± SEM ($n = 5$) of measurements from three independent experiments. The *inset* shows the PEG-ZnPP structure. *, $p < 0.05$ vs PEG-ZnPP-treated groups (unpaired Student's t test). *B*, Bacterial growth in livers from PEG-ZnPP-treated and control mice was measured via the colony formation assay at day 5 after infection. Data are means ± SEM ($n = 5$–7). *, $p < 0.05$ vs controls (unpaired Student's t test).

doses. The effect of inhibited HO-1 activity was associated with significantly enhanced bacterial yield (greater number of bacteria) in PEG-ZnPP-treated mice compared with controls at all bacterial doses (Fig. 5*B*; $p < 0.05$).

A similar effect of PEG-ZnPP treatment on bacterial growth was observed in vitro. Fig. 6, *A* and *B*, show that at each time point after infection, 6, 12, and 24 h, the number of bacteria in PEG-ZnPP-treated RAW 264 cells and peritoneal macrophages was significantly higher than that in controls (no PEG-ZnPP treatment; $p < 0.01$).

We also evaluated intracellular bacterial growth in PEG-ZnPP-treated and -untreated peritoneal macrophages in culture via microscopic methods. *S. typhimurium* organisms were immunostained with FITC-conjugated anti-*Salmonella* Ab at 24 h after infection. PEG-ZnPP-treated macrophages showed greater fluorescence intensity compared with untreated cells (Fig. 6*C*). Thus,

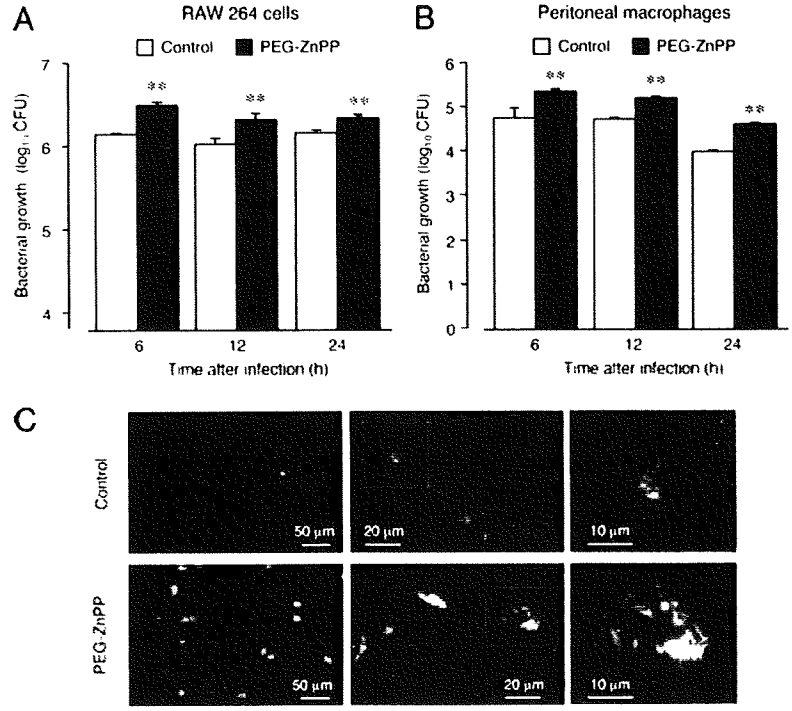

FIGURE 6. Effects of HO-1 inhibition by PEG-ZnPP on growth of bacteria in cultured macrophages. RAW 264 cells (*A*) and mouse peritoneal macrophages (*B*) were seeded at densities of 2×10^5 cells/well and 5×10^5 cells/well, respectively, in 24-well plates and were infected with *S. typhimurium* at 10 MOI. Cells were treated or not with 20 μM PEG-ZnPP to inhibit HO-1. Bacterial growth at 6, 12, and 24 h after infection was measured by means of the colony formation assay. Data are means ± SD of triplicate wells. **, $p < 0.01$ vs controls (unpaired Student's t test). *C*, Immunostaining for *Salmonella* in PEG-ZnPP-treated and -untreated peritoneal macrophages. Peritoneal macrophages were infected with *S. typhimurium* at 10 MOI and were treated with 20 μM PEG-ZnPP at 12 h before infection and 1 h after gentamicin treatment. Intracellular growth of *Salmonella* at 24 h was visualized by immunostaining with anti-*Salmonella*-FITC Ab.

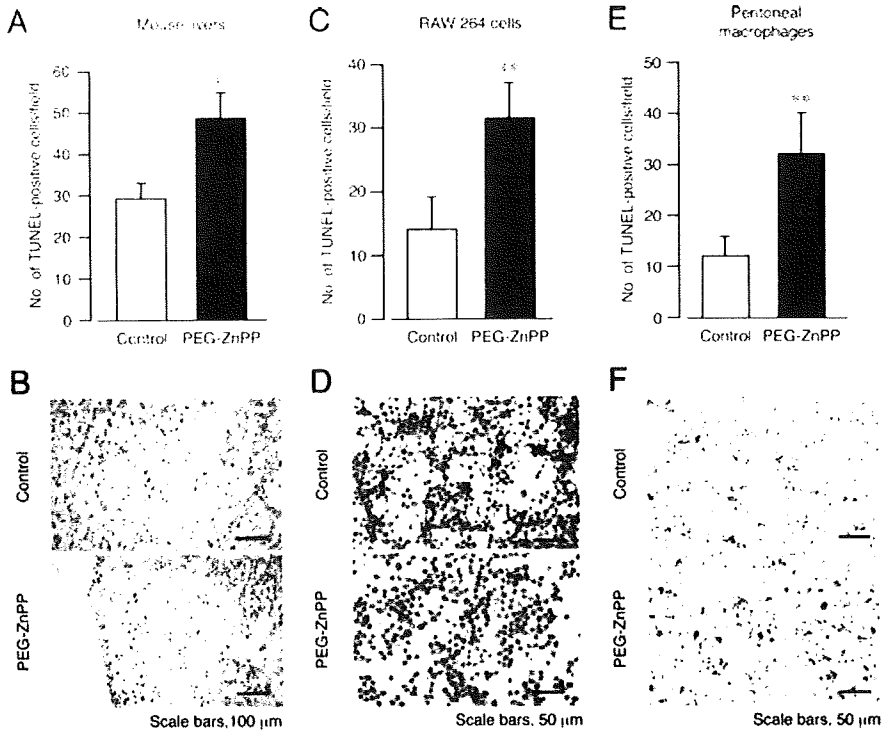

FIGURE 7. Induction of apoptosis after PEG-ZnPP treatment of *Salmonella*-infected mouse livers and cultured macrophages. *A*, Wild-type mice were infected with *S. typhimurium* at a dose of 2×10^4 CFU/mouse and treated or not with PEG-ZnPP. The TUNEL assay of liver tissues collected at day 5 after infection provided a measure of apoptosis. Morphometric analysis of TUNEL-positive cells was performed with five fields in two different sections from each mouse (three mice per group). Data are means ± SEM of measurements from two different experiments. *, $p < 0.05$ vs no PEG-ZnPP (control; unpaired Student's *t* test). *B*, Representative TUNEL results for data shown in *A* for PEG-ZnPP-treated and -untreated mouse livers. Dark blue identifies TUNEL-positive cells. RAW 264 cells (*C*) seeded at a density of 2×10^5 cells/well and thioglycolate-elicited peritoneal macrophages from wild-type mice (*E*) seeded at a density of 5×10^5 cells/well were infected with *S. typhimurium* at 10 MOI. Cells were treated or not with 20 μM PEG-ZnPP. Apoptosis was determined via the TUNEL assay at 12 h after infection. Data are means ± SD of 25 different microscopic fields from two independent experiments. **, $p < 0.01$ vs no PEG-ZnPP (control; unpaired Student's *t* test). *D* and *F*, Representative TUNEL results for data shown in *C* and *E* for PEG-ZnPP-treated and -untreated cells.

inhibition of HO-1 activity enhanced susceptibility to *Salmonella* infection, which suggests the potential involvement of HO-1 in defense of macrophages against *Salmonella*.

Furthermore, we examined, via TUNEL analysis, apoptotic cell death occurring in liver tissues from PEG-ZnPP-treated and -untreated mice. Liver samples were collected at day 5 after infection with *S. typhimurium* at a dose of 2×10^4 CFU/mouse. Fig. 7, *A* and *B*, demonstrates significantly more TUNEL-positive cells in PEG-ZnPP-treated mouse livers than in untreated mouse livers ($p < 0.05$). Most TUNEL-positive cells were confined to the area of microabscesses, which were composed of infiltrated macrophages, neutrophils, and degenerated hepatocytes. This finding indicates that inhibition of HO-1 activity by PEG-ZnPP treatment during *S. typhimurium* infection may accelerate tissue injury and cell death, which in turn would facilitate bacterial growth and histopathological changes in infected liver.

We evaluated the effects of PEG-ZnPP treatment on apoptosis in vitro by in situ TUNEL analysis with *Salmonella*-infected (10 MOI) RAW 264 cells and macrophages at 12 h after infection. Both RAW 264 cells (Fig. 7, *C* and *D*) and peritoneal macrophages (Fig. 7, *E* and *F*) had significantly ($p < 0.01$) higher levels of apoptosis after PEG-ZnPP treatment compared with untreated controls. As a control compound for PEG-ZnPP, we used PEG-PP, which lacks the zinc ion and thus HO inhibitory activity. PEG-PP had no significant effect on apoptosis induction and intracellular growth of *Salmonella* in RAW 264 cells (data not shown).

Results obtained by pharmacological inhibition of HO-1 were also confirmed by an alternative approach involving the use of siRNA for HO-1. Peritoneal macrophages from wild-type mice were treated with either HO-1 siRNA or control siRNA for 48 h, after which cells were infected with *Salmonella* at 10 MOI. Bacterial growth was measured at 6 and 18 h after infection, and apoptosis was measured by TUNEL analysis at 15 and 24 h after infection. Transfection of macrophages with HO-1 siRNA caused a significantly reduced expression of HO-1 protein as detected by Western blotting (Fig. 8*A*). HO-1 siRNA-treated cells had a significantly higher bacterial count (Fig. 8*B*) and apoptosis (Fig. 8*C*) compared with control siRNA-treated cells. These results therefore support the findings obtained with PEG-ZnPP and suggest that HO-1 is protective in *S. typhimurium* infection.

All of these findings together suggest that HO-1 plays a critical role in host defense during murine salmonellosis.

Cytoprotective effect of 8-nitro-cGMP

Because 8-nitro-cGMP is involved in HO-1 induction and HO-1 has a cytoprotective function during *Salmonella* infection, we investigated whether 8-nitro-cGMP has a direct cytoprotective effect. $iNOS^{-/-}$ macrophages were treated with 30 μM 8-nitro-cGMP at 6 h after *Salmonella* infection. As shown in Fig. 9*A*, higher apoptosis was observed in $iNOS^{-/-}$ macrophages compared with wild-type macrophages, but the difference was nullified when $iNOS^{-/-}$ cells were treated with 8-nitro-cGMP. No apparent

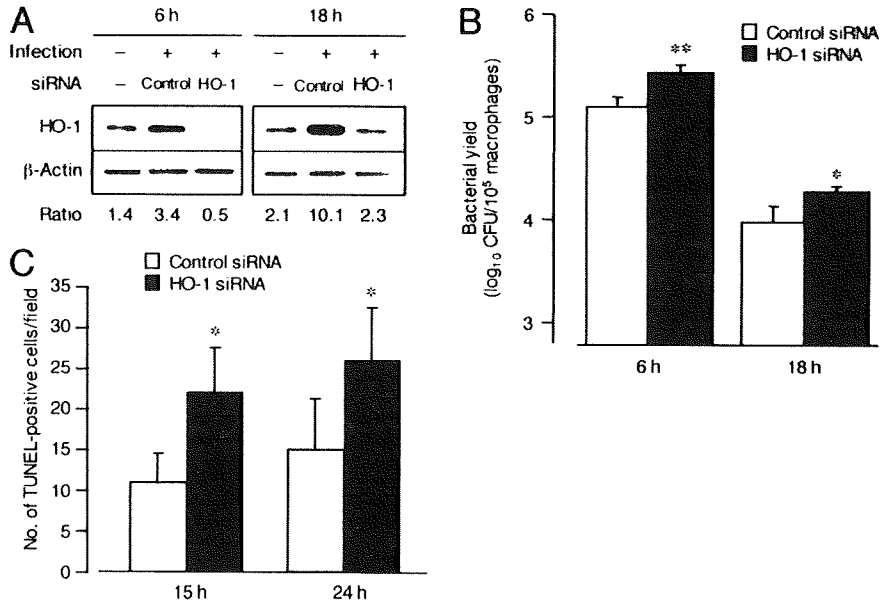

FIGURE 8. Effect of HO-1 inhibition by siRNA on bacterial growth and cytoprotection. *A*, Peritoneal macrophages from wild-type mice were transfected with either HO-1 siRNA or control siRNA at a final concentration of 100 nM. Cells were infected with *S. typhimurium* at 10 MOI at 48 h after transfection. HO-1 gene knockdown was assessed by Western blot analysis. *B*, Bacterial growth in siRNA-treated macrophages just mentioned was measured after infection by means of the colony formation assay. Data are means ± SD ($n = 3$). *, $p < 0.05$ and **, $p < 0.01$ vs control siRNA (unpaired Student's t test). *C*, Apoptosis was measured via the TUNEL analysis at 15 and 24 h after infection. Data are means ± SD of 40 microscopic fields. *, $p < 0.05$ vs control siRNA (unpaired Student's t test).

cytoprotection was observed for 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP), a widely used cGMP analog, which indicates that the cytoprotective effect of 8-nitro-cGMP occurred via a pathway independent of classical cGMP signal transduction. The apoptotic index was also measured by means of caspase 3 activation at 24 h after infection and was detected by Western blot analysis of active caspase 3 in *Salmonella*-infected macrophages. Consistent with TUNEL analysis results, higher caspase 3 activation was found in iNOS$^{-/-}$ macrophages compared with wild-type cells, and this activation was suppressed by 8-nitro-cGMP treatment (Fig. 9*B*). Release of cytochrome *c* was used to investigate the upstream pathway of the caspase cascade. iNOS$^{-/-}$ macrophages had showed greater mitochondrial release of cytochrome *c* in cytosol compared with wild-type macrophages (Fig. 9*B*). This higher cytochrome *c* release was partially blocked by 8-nitro-cGMP treatment (Fig. 9*B*). HO-1 may have a direct or indirect role in this 8-nitro-cGMP-mediated inhibition of caspase 3 activation, because greater activation of caspase 3 occurred after inhibition of HO-1 by PEG-ZnPP (data not shown).

These findings further confirmed the hypothesis that 8-nitro-cGMP has a signaling function in NO-mediated host defense via induction of HO-1.

Discussion

Although NO-dependent host defense and cytoprotection have been documented in a series of studies of *S. typhimurium* infection in mice (2, 45–49), biochemical and molecular mechanisms of NO-mediated host defense, particularly cytoprotection, have not been clearly addressed. Our study here clarified that NO-dependent

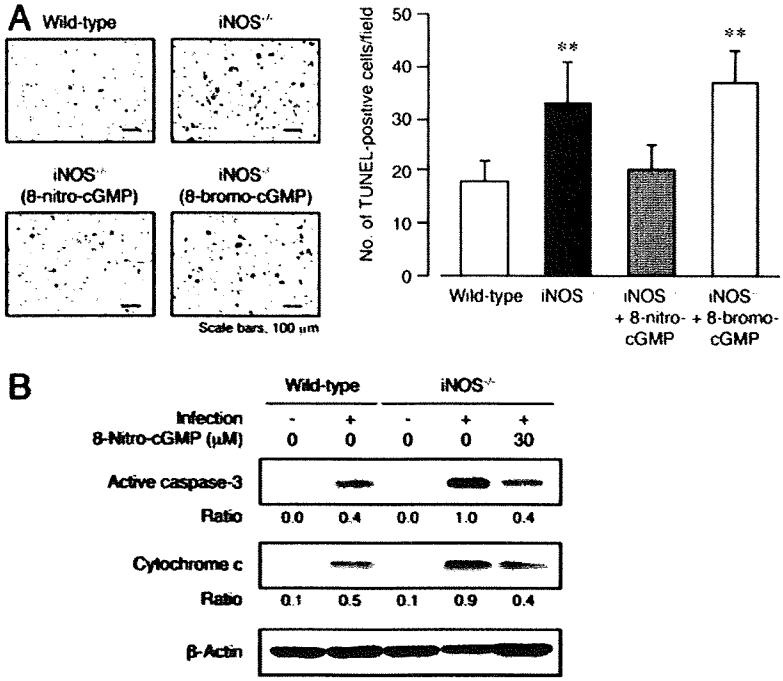

FIGURE 9. Cytoprotective effect of 8-nitro-cGMP. *A*, Peritoneal macrophages from wild-type and iNOS$^{-/-}$ mice were infected with *S. typhimurium* at 10 MOI. At 6 h after infection, 30 μM 8-nitro-cGMP or 30 μM 8-bromo-cGMP was added to iNOS$^{-/-}$ macrophage cultures. Apoptosis was measured by TUNEL analysis at 24 h after infection. The numbers of apoptotic cells were expressed as means ± SD of 40 different microscopic fields (original magnification, ×160) from two independent experiments. **, $p < 0.01$ vs control wild-type (unpaired Student's t test). *B*, Peritoneal macrophages from wild-type and iNOS$^{-/-}$ mice were infected with *S. typhimurium* at 10 MOI. iNOS$^{-/-}$ macrophages were treated with 30 μM 8-nitro-cGMP or were untreated. Cell lysates collected at 24 h of infection were subjected to Western blot analysis for active caspase 3 and cytochrome *c*.

HO-1 induction is mediated, at least in part, by 8-nitro-cGMP and contribute to the host defense potential for *Salmonella* infection.

NO has diverse physiological and pathological functions during infection and inflammation. Its two opposite biological effects, i.e., cytoprotection and cytotoxicity, depend on the chemical reactivities of NO and RNS. For example, NO reacts with O_2^- and produces RNS such as $ONOO^-$ and NO_2. These species interact with biological molecules, including thiols, lipids, proteins, and nucleic acids via nitration reactions and thereby form nitrated derivatives (54, 55).

Our previous studies indicated that nitration of proteins and tyrosine occurs in a manner dependent on NO produced from iNOS during murine salmonellosis (2, 45, 49). More important, we recently observed that excessive production of NO mediates guanine nitration in influenza virus-infected mouse lung (10). Nitration of guanine and/or guanosine in an environment in which NO is produced was also recently verified in other inflammatory lung diseases in humans (17). We reported in our recent study that, among several derivatives of 8-nitroguanine, 8-nitro-cGMP was the major product in biological systems and possessed certain unique features such as electrophilic, redox active, and cell signaling potentials (20). Moreover, 8-nitro-cGMP reacted readily with cysteine sulfhydryl groups of proteins to form cysteine-cGMP adducts, this reaction being called *S*-guanylation (20). In that study, we demonstrated *S*-guanylation of Keap1, a cysteine-rich protein involving sequestration of transcription factor Nrf2 in cytosol of macrophages stimulated with LPS plus IFN-γ or with *Salmonella* infection. The reaction of 8-nitro-cGMP with Keap1 presumably dissociates Nrf2 from the Keap1-Nrf2 complex, which leads to its nucleic translocation and the subsequent transcriptional activation of antioxidant factors including HO-1.

We therefore hypothesized that 8-nitro-cGMP-mediated *S*-guanylation of Keap1 may produce a functional modification of the Keap1 protein. We also postulated that *S*-guanylation of Keap1 may induce transcription of HO-1 through an Nrf2 pathway as a major mechanism of NO-mediated HO-1 induction. It was thus of great importance to see whether 8-nitro-cGMP indeed formed during *Salmonella* infection and participated in HO-1 induction. In this study, we clearly demonstrated that 8-nitro-cGMP was formed in the pathophysiological condition of salmonellosis and that 8-nitro-cGMP possessed a strong potential for HO-1 induction. This study provides critical information in research exploring the formation and functional role of a newly identified nitrated cyclic nucleotide, 8-nitro-cGMP, in a pathophysiological context.

Under inflammatory conditions, electrophilic substances formed endogenously and potentially involved in the Keap1/Nrf2 pathway, include 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), a product of cyclooxygenase, and 4-hydroxy-2-nonenal (4-HNE). Keap1 cysteine modification by 15d-PGJ_2 was suggested to occur by using biotinylated derivatives of 15d-PGJ_2 added exogenously to cells (56). However, it is still unclear whether endogenous 15d-PGJ_2 can indeed modify Keap1. It was recently reported that intracellular concentrations of 15d-PGJ_2 formed endogenously is as low as 1 nM (57). Furthermore, cysteine adducts of 15d-PGJ_2 are so unstable to readily dissociate spontaneously under physiological conditions (58). These factors may hamper detection of Keap1-PGJ_2 adducts. Further study is needed to clarify the relative contribution of the iNOS-dependent 8-nitro-cGMP pathway and cyclooxygenase-dependent 15d-PGJ_2 pathway for activation of Keap1/Nrf2 signaling by developing a sensitive method to detect unstable Keap1-PGJ_2 adducts. 4-HNE is formed during the process of lipid peroxidation, and hence, is associated with oxidative stress (59). We observed that oxidant formation in macrophages during *Salmonella* infection was higher in iNOS-deficient cells than in wild-type cells (data not shown). This suggests that oxidative stress-associated production of 4-HNE may not be a major pathway to induce Keap1/Nrf2-dependent HO-1 induction in our murine salmonellosis model.

Earlier studies documented HO-1 induction by NO in various cultured cells (22–24), including macrophages treated with LPS (25), and in in vivo solid tumor models (26, 27), rat models of sepsis produced by LPS treatment (60, 61), and a mouse ischemia-reperfusion model (62). All of these data are thus consistent with our current investigations of murine salmonellosis. In contrast, however, Barreiro et al. (63) did not find NO-dependent HO-1 induction in diaphragm muscle of rats treated with LPS. They suggested that oxidative stress induced by LPS in muscle may be the major contributor to HO-1 induction. These discrepant results may be due to the different tissues and animal models used. In any event, macrophages are the primary cells harboring *Salmonella* and play a central part in innate host defense. Therefore, macrophage-specific induction of HO-1 up-regulated via NO production seems to have some significance in host defense.

We used two approaches to investigate the exact function of HO-1 induced by NO and 8-nitro-cGMP in our *Salmonella* infection models. One was enzyme inhibition by ZnPP IX, which is commonly used to examine the effect of HO-1 expression in experimental models. However, a potential problem with using ZnPP IX in such studies is its insolubility in water. To overcome this problem, we developed a water-soluble ZnPP IX derivative by conjugating it with PEG (Ref. 51; the *inset* in Fig. 5*A* shows its structure). The resultant PEG-ZnPP is highly water soluble, can be administered in vivo by the i.v. route, and is as effective as ZnPP IX in inhibiting HO-1 (41, 42, 51). Also, we found PEG-ZnPP to be more effective than ZnPP in targeted inhibition of HO-1 in solid tumors because it demonstrated an increased retention time in tissues, which was a result of its being a macromolecule (41, 42). In addition, increased PEG-ZnPP accumulated in liver and spleen without affecting the physiological functions of these organs (41, 42). In fact, in our present study, PEG-ZnPP treatment effectively inhibited HO-1 activity, in that PEG-ZnPP suppressed the blood CO level in *S. typhimurium*-infected mice (Fig. 5*A*). The other approach that we used was HO-1 gene knockdown by siRNA. As Fig. 8 illustrates, the trends of increased apoptosis and greater bacterial growth after inhibition of HO-1 by siRNA support the finding of a host defense function of HO-1 as shown by studies using PEG-ZnPP just mentioned.

Catalytic products of HO-1 reaction have been known to possess cytoprotective and antiapoptotic activities. Bilirubin is formed as a result of intracellular reduction of biliverdin by biliverdin reductase (32, 33). Bilirubin thus formed is known as a potent antioxidant (32, 33). ROS and RNS produced by phagocytes after stimulation with particular pathogens play a role in elimination of phagocytosed bacteria, but also are cytotoxic to the phagocytes themselves, particularly at high concentrations. Bilirubin may protect phagocytes from ROS/RNS-induced oxidative cellular damage. In addition, another HO product, CO possesses cytoprotective and antiapoptotic activities. Accordingly, up-regulation of HO-1 protects macrophages from apoptosis induced by *Salmonella* infection, thereby facilitating the killing of *Salmonella* organisms. In fact, our preliminary study performed in a separate experiment indicates that HO-1 induced by 8-nitro-cGMP formed endogenously in the cells could exert potent antioxidant and cytoprotective functions in cells in culture (our unpublished observation). On the other hand, Nobre et al. (64) recently demonstrated that exogenous CO can inhibit growth of pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* (64). Thus, cytoprotection by HO-1 may be mediated by suppression of bacterial growth as an

alternative possibility. The contribution of HO-1-derived CO on the antimicrobial effect should be clarified in the future study.

We observed in this study that HO-1 was induced in iNOS$^{-/-}$ mice/macrophages during *Salmonella* infection, although the level of HO-1 was much lower in iNOS$^{-/-}$ cells than in wild-type cells. It has been reported that HO-1 can be induced in response to various stimuli, including ROS (29) and bacterial component LPS (24, 25). We found that *Salmonella* infection induced ROS generation in both wild-type and iNOS$^{-/-}$ macrophages; the level was slightly higher in iNOS$^{-/-}$ macrophages (data not shown). Therefore, it can be assumed that ROS and LPS may contribute to the induction of HO-1 in iNOS$^{-/-}$ cells during *Salmonella* infection.

One of the reasons for lethality in murine salmonellosis is septic shock (2, 45). The pathogenesis of sepsis is characterized by pathological events caused by uncontrolled production of proinflammatory cytokines (such as TNF-α and IL-1β) and macrophage inflammatory proteins, which would lead to leukocyte recruitment, capillary leaking, and tissue damage, and ultimately lethality (39, 65). Recent studies have suggested that HO-1 inhibits production of proinflammatory cytokines (66). We therefore measured the TNF-α level in *S. typhimurium*-infected mouse serum and culture supernatant of peritoneal macrophages with or without PEG-ZnPP treatment. We found a markedly higher TNF-α level in PEG-ZnPP-treated mouse serum than in untreated mouse serum after *S. typhimurium* infection (data not shown). We obtained a similar result in an in vitro study of cultured infected peritoneal macrophages (data not shown). HO-1 may thus have anti-inflammatory activity during *S. typhimurium* infection by inhibiting proinflammatory cytokines such as TNF-α.

In summary, this study described a unique mechanism of NO-mediated host defense in which NO causes formation of a novel signaling molecule, 8-nitro-cGMP, which possesses several important biological functions including induction of HO-1. This study also demonstrated that HO-1 induced by 8-nitro-cGMP plays an important role in innate immunity by protecting cells from apoptosis and possibly by promoting phagocytic bacterial killing by macrophages. The up-regulation of HO-1 induced by NO and involvement of HO-1 in host defense may aid understanding of the molecular mechanisms of NO-mediated host defense (67). It would be extremely interesting to explore other target proteins for *S*-guanylation by 8-nitro-cGMP during NO-mediated host defense. Additional studies of host defense activities mediated by 8-nitro-cGMP and HO-1 in other microbial infections are thus warranted.

Acknowledgments

We thank Judith B. Gandy for excellent editing of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Nathan, C. F., and J. B. Hibbs, Jr. 1991. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* 3: 65–70.
2. Umezawa, K., T. Akaike, S. Fuji, M. Suga, K. Setoguchi, A. Ozawa, and H. Maeda. 1997. Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against *Salmonella typhimurium* infection in mice. *Infect. Immun.* 65: 2932–2940.
3. Mannick, J. B., X. Q. Miao, and J. S. Stamler. 1997. Nitric oxide inhibits Fas-induced apoptosis. *J. Biol. Chem.* 272: 24125–24128.
4. Kim, Y. M., M. E. Vera, S. C. Watkins, and T. R. Billiar. 1997. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-α-induced apoptosis by inducing heat shock protein 70 expression. *J. Biol. Chem.* 272: 1402–1411.
5. Wei, X. Q., I. G. Charles, A. Smith, J. Ure, G. J. Feng, F. P Huang, D. Xu, W. Muller, S. Moncada, and F. Y. Liew. 1995. Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature* 375: 408–411.
6. Nathan, C., and M. U. Shiloh. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc. Natl. Acad. Sci. USA* 97: 8841–8848.
7. Beckman, J. S., and W. H. Koppenol. 1996. Nitric oxide, superoxide and peroxynitrite: the good, the bad, and ugly. *Am. J. Physiol.* 271: C1424–C1437.
8. Eiserich, J. P., M. Hristova, C. E. Cross, A. D. Jones, B. A. Freeman, B. Halliwell, and A. van der Vliet. 1998. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397.
9. Sawa, T., T. Akaike, and H. Maeda. 2000. Tyrosine nitration by peroxynitrite formed from nitric oxide and superoxide generated by xanthine oxidase. *J. Biol. Chem.* 275: 32467–32474.
10. Akaike, T., S. Okamoto, T. Sawa, J. Yoshitake, F. Tamura, K. Ichimori, K. Miyazaki, K. Sasamoto, and H. Maeda. 2003. 8-Nitroguanosine formation in viral pneumonia and its implication for pathogenesis. *Proc. Natl. Acad. Sci. USA* 100: 685–690.
11. Haddad, I. Y., G. Pataki, P. Hu, C. Galliani, J. S. Beckman, and S. Matalon. 1994. Quantification of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *J. Clin. Invest.* 94: 2407–2413.
12. Hernandez-Pando, R., T. Schon, E. H. Orozco, J. Serafin, and I. Estrada-Garcia. 2001. Expression of inducible nitric oxide synthase and nitrotyrosine during the evolution of experimental pulmonary tuberculosis. *Exp. Toxicol. Pathol.* 53: 257–265.
13. Akaike, T., Y. Noguchi, S. Ijiri, K. Setoguchi, M. Suga, Y. M. Zheng, B. Dietzschold, and H. Maeda. 1996. Pathogenesis of influenza virus-induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc. Natl. Acad. Sci. USA* 93: 2448–2453.
14. Yermilov, V., J. Rubio, and H. Ohshima. 1995. Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett.* 376: 207–210.
15. Masuda, M., H. Nishino, and H. Oshima. 2002. Formation of 8-nitroguanosine in cellular RNA as a biomarker of exposure to reactive nitrogen species. *Chem. Biol. Interact.* 139: 187–197.
16. Byun, J., J. P. Henderson, D. M. Mueller, and J. W. Heinecke. 1999. 8-Nitro-2′-deoxyguanosine, a specific marker of oxidation by reactive nitrogen species, is generated by the myeloperoxidase-hydrogen peroxide-nitrite system of activated human phagocytes. *Biochemistry* 38: 2590–2600.
17. Terasaki, Y., T. Akuta, M. Terasaki, T. Sawa, T. Mori, T. Okamoto, M. Ozaki, M. Takeya, and T. Akaike. 2006. Guanine nitration in idiopathic pulmonary fibrosis and its implication for carcinogenesis. *Am. J. Respir. Crit. Care Med.* 174: 665–673.
18. Yoshitake, J., T. Akaike, T. Akuta, F. Tamura, T. Ogura, H. Esumi, and H. Maeda. 2004. Nitric oxide as an endogenous mutagen for Sendai virus without antiviral activity. *J. Virol.* 78: 8709–8719.
19. Sawa, T., T. Akaike, K. Ichimori, T. Akuta, K. Kaneko, H. Nakayama, D. J. Stuehr, and H. Maeda. 2003. Superoxide generation mediated by 8-nitroguanosine, a highly redox active nucleic acid derivative. *Biochem. Biophys. Res. Commun.* 311: 300–306.
20. Sawa, T., M. H. Zaki, T. Okamoto, T. Akuta, Y. Tokutomi, S. Kim-Mitsuyama, H. Ihara, A. Kobayashi, M. Yamamoto, S. Fujii, H. Arimoto, and T. Akaike. 2007. Protein S-guanylation by the biological signal 8-nitroguanosine 3′,5′-cyclic monophosphate. *Nat. Chem. Biol.* 3: 727–735.
21. Maines, M. D. 1988. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J.* 2: 2557–2568.
22. Choi, B. M., H. O. Pae, Y. M. Kim, and H. T. Chung. 2003. Nitric oxide-mediated cytoprotection of hepatocytes from glucose deprivation-induced cytotoxicity: involvement of heme oxygenase-1. *Hepatology* 37: 810–823.
23. Motterlini, R., R. Foresti, M. Intaglietta, and R. M. Winslow. 1996. NO-mediated activation of heme oxygenase: endogenous cytoprotection against oxidative stress to endothelium. *Am. J. Physiol.* 270: H107–H114.
24. Immenschuh, S., M. Tan, and G. Ramadori. 1999. Nitric oxide mediates the lipopolysaccharide dependent upregulation of the heme oxygenase-1 gene expression in cultured rat Kupffer cells. *J. Hepatol.* 30: 61–69.
25. Ashino, T., R. Yamanaka, M. Yamamoto, H. Shimokawa, K. Sekikawa, Y. Iwakura, S. Shioda, S. Numazawa, and T. Yoshida. 2008. Negative feedback regulation of lipopolysaccharide-induced inducible nitric oxide synthase gene expression by heme oxygenase-1 induction in macrophages. *Mol. Immunol.* 45: 2106–2115.
26. Doi, K., T. Akaike, S. Fujii, S. Tanaka, N. Ikebe, T. Beppu, S. Shibahara, M. Ogawa, and H. Maeda. 1999. Induction of heme oxygenase-1 by nitric oxide and ischemia in experimental solid tumors and implications for tumor growth. *Br. J. Cancer* 80: 1945–1954.
27. Tanaka, S., T. Akaike, J. Fang, M. Ogawa, F. Tamura, Y. Miyamoto, and H. Maeda. 2003. Antiapoptotic effect of haem oxygenase-1 induced by nitric oxide in experimental solid tumor. *Br. J. Cancer* 88: 902–909.
28. Otterbein, L., S. L. Sylvester, and A. M. Choi. 1995. Hemoglobin provides protection against lethal endotoxemia in rats: the role of heme oxygenase-1. *Am. J. Respir. Cell Mol. Biol.* 13: 595–601.
29. Keyse, S. M., and R. M. Tyrrell. 1989. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide and sodium arsenite. *Proc. Natl. Acad. Sci. USA* 86: 99–103.
30. Mitani, K., H. Fujita, Y. Fukuda, A. Kappas, and S. Sassa. 1993. The role of inorganic metals and metalloporphyrins in the induction of heme oxygenase and heat shock protein 70 in human hepatoma cells. *Biochem. J.* 290: 819–825.
31. Shibahara, S., R. Millar, and H. Taguchi. 1987. Transcriptional control of rat heme oxygenase by heat shock. *J. Biol. Chem.* 262: 12889–12892.
32. Baranano, D. E., M. Rao, C. D. Ferris, and S. H. Snyder. 2002. Biliverdin reductase: a major physiologic cytoprotectant. *Proc. Natl. Acad. Sci. USA* 99: 15837–15839.

33. Stocker, R., Y. Yamamoto, A. F. McDonagh, A. N. Glazer, and B. N. Ames. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* 235: 1043–1046.
34. Motterlini, R., A. Gonzales, R. Foresti, J. E. Clark, C. J. Green, and R. M. Winslow. 1998. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive response in vivo. *Circ. Res.* 83: 568–577.
35. Suematsu, M., N. Goda, T. Sano, S. Kashiwagi, T. Egawa, Y. Shinoda, and Y. Ishimura. 1995. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J. Clin. Invest.* 96: 2431–2437.
36. Brouard, S., L. E. Otterbein, J. Anrather, E. Tobiasch, F. H. Bach, A. M. Choi, and M. P. Soares. 2000. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J. Exp. Med.* 192: 1015–1026.
37. Zuckerbraun, B. S., T. R. Billiar, S. L. Otterbein, P. K. Kim, F. Liu, A. M. Choi, F. H. Bach, and L. E. Otterbein. 2004. Carbon monoxide protects against liver failure through nitric oxide-induced heme oxygenase 1. *J. Exp. Med.* 198: 1707–1716.
38. Sass, G., M. C. Soares, K. Yamashita, S. Seyfried, W. H. Ziemmermann, T. Eschenhagen, E. Kaczmarek, T. Ritter, H. D. Volk, and G. Tiegs. 2003. Heme oxygenase-1 and its reaction product, carbon monoxide, prevent inflammation-related apoptotic liver damage in mice. *Hepatology* 38: 909–918.
39. Otterbein, L. E., F. H. Bach, J. Alam, M. Soares, H. T. Lu, M. Wysk, R. J. Davis, R. A. Flavell, and A. M. K. Choi. 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat. Med.* 6: 422–428.
40. Song, R., R. S. Mahidhara, Z. Zhou, R. A. Hoffman, D. W. Seol, R. A. Flavell, T. R. Billiar, L. E. Otterbein, and A. M. K. Choi. 2004. Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. *J. Immunol.* 172: 1220–1226.
41. Fang, J., T. Sawa, T. Akaike, T. Akuta, S. K. Sahoo, G. Khaled, A. Hamada, and H. Maeda. 2003. In vitro antitumor activity of pegylated zinc protoporphyrin: targeted inhibition of heme oxygenase in solid tumor. *Cancer Res.* 63: 3563–3574.
42. Fang, J., T. Sawa, T. Akaike, G. Khaled, and H. Maeda. 2004. Enhancement of chemotherapeutic response of tumor cells by a heme oxygenase inhibitor, pegylated zinc protoporphyrin. *Int. J. Cancer.* 109: 1–8.
43. MacMicking, J. D., R. J. North, R. LaCourse, J. S. Mudgett, S. K. Shah, and C. F. Nathan. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. USA* 94: 5243–5248.
44. Shiloh, M. U., and C. F. Nathan. 2000. Reactive nitrogen intermediates and the pathogenesis of *Salmonella* and mycobacteria. *Curr. Opin. Microbiol.* 3: 35–42.
45. Alam, M. S., T. Akaike, S. Okamoto, T. Kubota, J. Yoshitake, T. Sawa, Y. Miyamoto, F. Tamura, and H. Maeda. 2002. Role of nitric oxide in host defense in murine salmonellosis as a function of its antimicrobial and antiapoptotic activities. *Infect. Immun.* 70: 3130–3142.
46. Alam, M. S., M. H. Zaki, J. Yoshitake, T. Akuta, T. Ezaki, and T. Akaike. 2006. Involvement of *Salmonella enterica* serovar Typhi RpoS in resistance to NO-mediated host defense against serovar Typhi infection. *Microb. Pathog.* 40: 116–125.
47. Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche, and G. Dougan. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis: II. Effects on microbial proliferation and host survival in vivo. *J. Exp. Med.* 192: 237–248.
48. Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ichiropoulos, and F. C. Fang. 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis: I. Effects on microbial killing by activated peritoneal macrophages in vitro. *J. Exp. Med.* 192: 227–236.
49. Alam, M. S., M. H. Zaki, T. Sawa, S. Islam, K. A. Ahmed, S. Fujii, T. Okamoto, and T. Akaike. 2008. Nitric oxide produced in Peyer's patches exhibits antiapoptotic activity contributing to an antimicrobial effect in murine salmonellosis. *Microbiol. Immunol.* 52: 197–208.
50. MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. W. Xie, K. Sokol, N. Hutchinson, et al. 1995. Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81: 641–650.
51. Sahoo, S. K., T. Sawa, J. Fang, S. Tanaka, Y. Miyamoto, T. Akaike, and H. Maeda. 2002. Pegylated zinc protoporphyrin: a water-soluble heme oxygenase inhibitor with tumor-targeting capacity. *Bioconj. Chem.* 13: 1031–1038.
52. Vazquez-Torres, A., and F. C. Fang. 2001. Oxygen-dependent anti-*Salmonella* activity of macrophages. *Trends Microbiol.* 9: 29–33.
53. Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer, and C. Nathan. 1999. Phenotype of mice and macrophages deficient in both phagocytic oxidase and inducible nitric oxide synthase. *Immunity* 10: 29–38.
54. Beckman, J. S., and J. P. Crow. 1993. Pathological implications of nitric oxide, superoxide and peroxynitrite formation. *Biochem. Soc. Trans.* 21: 330–334.
55. Zaki, M. H., T. Akuta, and T. Akaike. 2005. Nitric oxide-induced nitrative stress involved in microbial pathogenesis. *J. Pharmacol. Sci.* 98: 117–129.
56. Oh, J. Y., N. Giles, A. Landar, and V. Darley-Usmar. 2008. Accumulation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 adduct formation with Keap1 over time: effects on pontecy for intracellular antioxidant defense induction. *Biochem. J.* 411: 297–306.
57. Bell-Parikh, L. C., T. Ide, J. A. Lawson, P. McNamara, M. Reilly, and G. A. FitzGerald. 2003. Biosynthesis of 15-deoxy-$\Delta^{12,14}$-PGJ_2 and the ligation of PPARg. *J. Clin. Invest.* 112: 945–955.
58. Suzuki, M., M. Mori, T. Niwa, R. Hirata, K. Furuta, T. Ishikawa, and R. Noyori. 1997. Chemical implications for antitumor and antiviral prostaglandins: reaction of Δ^7-prostaglandin A_1 and prostaglandin A_1 methyl esters with thiols. *J. Am. Chem. Soc.* 119: 2376–2385.
59. Uchida, K. 2003. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress Lipid Res.* 42: 318–343.
60. Taille, C., R. Foresti, S. Lanone, C. Zedda, C. Green, M. Aubier, R. Motterlini, and J. Boczkowski. 2001. Protective role of heme oxygenases against endotoxin-induced diaphragmatic dysfunction in rats. *Am. J. Respir. Crit. Care Med.* 163: 753–761.
61. Boczkowski, J., S. Lanone, D. Ungureanu-Longrois, G. Danialou, T. Fournier, and M. Aubier. 1996. Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats: role on diaphragmatic contractile dysfunction. *J. Clin. Invest.* 98: 1550–1559.
62. Zamora, R., Y. Vodovotz, K. S. Aulak, P. K. Kim, J. M. Kane III, L. Alarcon, D. J. Stuehr, and T. R. Billiar. 2002. A DNA microarray study of nitric oxide-induced genes in mouse hepatocytes: implications for hepatic heme oxygenase-1 expression in ischemia/reperfusion. *Nitric Oxide* 7: 165–186.
63. Barreiro, E., A. S. Comtois, S. Mohammad, L. C. Lands, and S. N. Hussain. 2002. Role of heme oxygenase in sepsis-induced diaphragmatic contractile dysfunction and oxidative stress. *Am. J. Physiol.* 283: L476–L484.
64. Nobre, L. S., J. D. Seixas, C. C. ROmao, and L. M. Saraiva. 2007. Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob. Agents Chemother.* 51: 4303–4307.
65. Clark, I. A., M. M. Awburn, C. G. Harper, N. G. Liomba, and M. E. Molyneux. 2003. Induction of HO-1 in tissue macrophages and monocytes in fatal falciparum malaria and sepsis. *Malar. J.* 2: 41.
66. Lee, T. S., and L. Y. Chau. 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat. Med.* 8: 240–246.
67. Bogdan, C., M. Rollinghoff, and A. Diefenbach. 2000. The role of nitric oxide in innate immunity. *Immunol. Rev.* 173: 17–26.

Seminars in Immunology 21 (2009) 78–83

Contents lists available at ScienceDirect

Seminars in Immunology

journal homepage: www.elsevier.com/locate/ysmim

Review

Critical role of the Polycomb and Trithorax complexes in the maintenance of CD4 T cell memory

Toshinori Nakayama*, Masakatsu Yamashita

Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

ARTICLE INFO

Keywords:
Th memory
Polycomb
Trithorax
Transcriptional memory

ABSTRACT

The maintenance of memory CD4 T cells is crucial for the establishment of immunological memory. The Polycomb (PcG) and Trithorax group (TrxG) genes control key developmental regulators such as the homeobox genes, and these two antagonize each other in the same developmental processes. Recently, PcG gene *Bmi1* has been found to control memory Th1/Th2 cell survival and TrxG gene *MLL* is to control the maintenance of memory Th2 cell function selectively. Therefore, in memory CD4 T cells, PcG and TrxG genes appear to control distinct processes in a distinct manner, which indicates a novel regulatory feature of the PcG/TrxG genes.

1. Introduction

After antigen recognition by TCR, naïve CD4 T cells undergo clonal expansion and become functionally polarized effector Th cells, such as Th1 and Th2 cells within a few weeks. After antigen clearance, however, most of the effector Th1/Th2 cells are thought to undergo apoptotic cell death during a period known as the contraction phase [1,2]. Some of the effector cells, however, escape cell death, differentiate into memory type Th1/Th2 cells and survive for a long time *in vivo* (memory phase) [3–5]. Various cellular and molecular processes are required for the successful differentiation and maintenance of functional memory type Th1/Th2 cells; cell survival/escape from cell death and proliferation/homeostatic proliferation at both contraction and memory phases, and the maintenance of Th1/Th2 cell function at memory phase [5]. The idea of memory stem cells is also considered, but this has not been experimentally addressed very well.

The Polycomb group (PcG) gene products are reported to localize in the nucleus as heterogeneous multimeric protein complexes and they appear to maintain, through silencing mechanisms, early determined gene expression patterns of key developmental regulators such as the homeobox genes both in invertebrates and vertebrates (reviewed in [6,7]). The PcG gene *Bmi1* has recently been implicated in the maintenance of hematopoietic [8,9], neural [10] and cancer stem cells [11]. The Trithorax group (TrxG) gene products are known to antagonize the effect of PcG gene products in the early developmental processes, and control nuclear regulatory mechanisms that establish the epigenetic transcriptional memory [12].

This review focuses on the recently identified molecular mechanisms by which PcG and TrxG gene products directly control the differentiation and the maintenance of functional memory CD4 T cells. A distinct view of the mode of regulation by PcG/TrxG gene products from that in the early developmental processes has emerged.

2. Establishment of an experimental system for the molecular analysis of memory Th cells

One of the difficulties in studying the molecular events operating in memory Th cells is the limitations in the preparation of substantial numbers of antigen-specific memory Th cells generated *in vivo*. To overcome this issue, an *in vivo* experimental system was established in which antigen-specific memory Th1 and Th2 cells are generated and maintained quite efficiently [13]. In brief, naïve CD4 T cells from DO11.10 OVA-specific TCR transgenic (Tg) mice are stimulated with a specific OVA peptide plus APC for 5 days *in vitro* under either Th1 or Th2 culture conditions, and then transferred intravenously into normal syngeneic BALB/c or BALB/c *nu/nu* recipient mice (Fig. 1A). The transferred DO11.10 Tg T cells can be monitored by the staining with clonotypic KJ1 mAb, which is specific for a donor-derived TCR Tg T cells. A week after cell transfer of the effector Th2 cells into normal BALB/c mice, ~25% of the splenic CD4 T cells are donor derived [13]. The numbers of $KJ1^+$ cells are decreased to approximately 10% at the 2 weeks time point, and this level is maintained at least for 16 weeks. Similar kinetics are

Abbreviations: PcG, Polycomb group; PRC, Polycomb repressive complex; TrxG, Trithorax group; Tg, transgenic.

* Corresponding author at: Department of Immunology (H3), Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel.: +81 43 226 2200; fax: +81 43 227 1498.
E-mail address: tnakayama@faculty.chiba-u.jp (T. Nakayama).

1044-5323/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.smim.2009.02.001

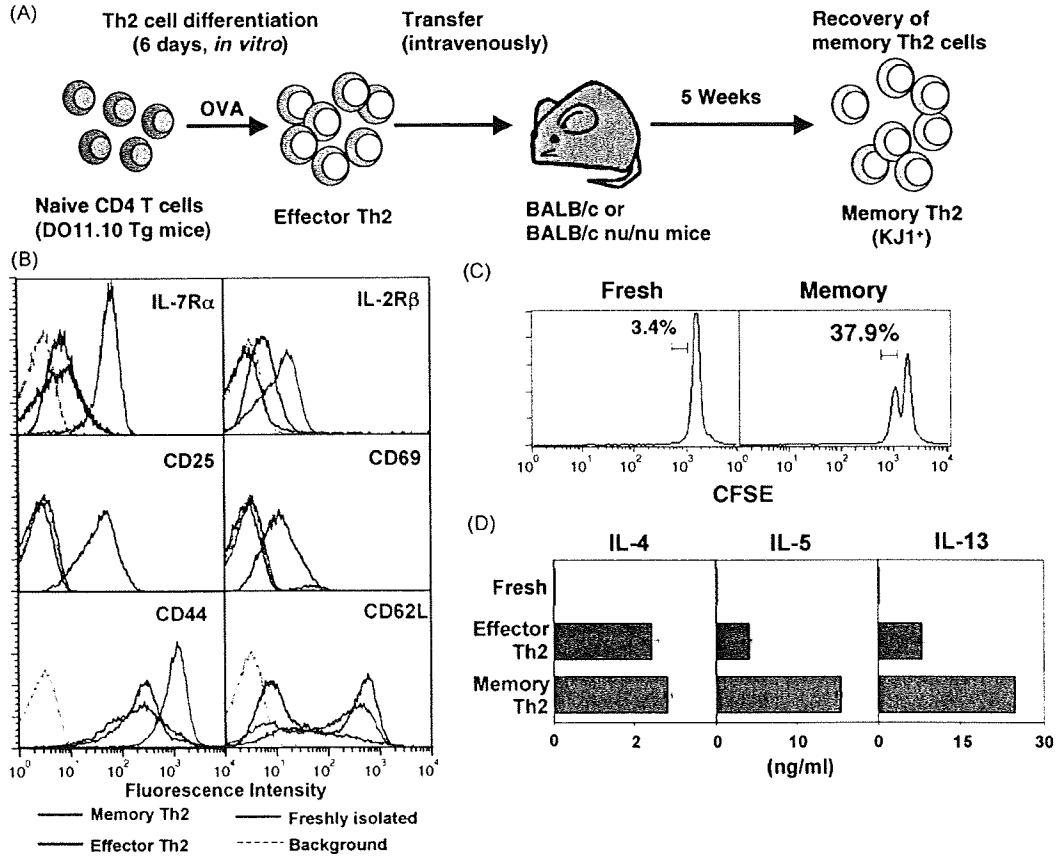

Fig. 1. Generation and phenotypic characterization of OVA-specific memory Th2 cells. (A) The generation of antigen-specific memory Th2 cells by adoptive cell transfer. Splenic CD4 T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15) plus APC under Th2 conditions for 5 days *in vitro*, and then transferred intravenously into normal syngeneic BALB/c or BALB/c *nu/nu* recipient mice. The donor-derived cells (memory Th2 cells) were identified by the staining with anti-clonotypic mAb for the Tg TCR, JK1. (B) Expression profiles of cell surface maker antigens on *in vivo* generated memory Th2 cells. (C) Memory Th2 cells rapidly proliferated in response to the antigen. The cells were labeled with CFSE and stimulated with OVA peptides plus APC for 16 h. The cell division of CFSE-labeled cells was analyzed by flow cytometry. (D) Th2 cyokine production profiles of the *in vivo* generated memory Th2 cells upon *in vitro* antigenic restimulation.

observed when effector Th2 cells are transferred into BALB/c *nu/nu* recipient mice (unpublished observation).

The expression profiles of the surface makers on the *in vivo* generated memory Th2 cells are shown in Fig. 1B. The expression levels of IL-7 receptor (R)α and IL-2 receptor (R)β chains are higher in memory Th2 cells in comparison to those of freshly isolated KJ1$^+$ CD4 T cells from DO11.10 Tg mice or *in vitro* generated effector Th2 cells. The activation markers, CD69 and CD25 (IL-2Rα chain), are not significantly expressed in either memory or naïve populations, whereas effector Th2 cells expressed these makers substantially. The high-level expression of CD44 is observed in the memory Th2 cells. Two subpopulations with high and low expression of CD62L (phenotypically central and effector memory cells, respectively) are observed in memory Th2 cells.

Memory T cells proliferate rapidly in response to a low concentration of antigens in comparison to naïve T cells [14]. *In vivo* generated memory Th2 cells from the spleen were labeled with CFSE *in vitro*, and stimulated with two different doses of OVA peptides and APC for 16 h. Freshly isolated CD4 T cells from DO11.10 Tg mice do not proliferate (3.4%) during the first 16 h after stimulation, whereas substantial numbers of memory Th2 cells (37.9%) divided once in response to the antigenic peptide (Fig. 1C). Similar results are obtained in Th1 cells (unpublished observation).

Cytokine production profiles are also maintained in the *in vivo* generated memory Th2 cells (Fig. 1D). After restimulation with antigenic peptide *in vitro*, the *in vivo* generated memory Th2 cells produced large amounts of Th2 cytokines (IL-4, IL-5 and IL-13) and the levels are equivalent or higher than those of effector Th2 cells.

Taken as a whole, these results indicate that the *in vivo* generated memory Th2 cells possess the phenotypic and functional properties that are typical for memory cells.

3. Molecular basis for the maintenance of memory CD4 T cells

In contrast to CD8 memory T cells, CD4 memory T cells may not require any specific cytokine signals for their homeostatic maintenance [15,16]. Memory CD4 T cells that lack common γ-chain (γc) could survive normally *in vivo* [17]. However, a role for IL-7 in memory CD4 T cells is demonstrated when the TCR-mediated signals are impaired [18]. Therefore, IL-7 may play a role in the regulation of the generation and survival of CD4 memory T cells [18–20]. The γc cytokines such as IL-7, IL-2, and IL-15 transduce signals through the recruitment of Jak1 and Jak3, which phosphorylate STAT5 [21]. The proto-oncogenes Pim1 and Pim2, which are transcriptional targets of STAT5, are preferentially expressed in the central memory T cell subset [22]. In addition, a higher level of STAT5 phosphorylation is induced by IL-2/IL-7-treatment in central memory T cells in comparison to effector memory T cells. These results suggest that central memory T cells are more sensitive to the γc cytokines to be activated and proliferate.

Recently, the upregulation of several target genes (Bim, Gadd45α, p130 proteins and Fas ligand) of the forkhead-family

transcription factor, FOXO3a was reported in $CD4^+$ effector memory T cells [23]. The activity of FOXO3a is suppressed by Akt- and IKK-mediated Ser/Thr-phosphorylation [24,25]. Stimulation via the TCR/CD28 and cytokines activated Akt through the PI3K-dependent signaling pathway and regulated phosphorylation of FOXO3. TCR/CD28-mediated signals induced phosphorylation on Ser315 of FOXO3a, whereas IL-2/IL-7 stimulation results in the phosphorylation on Thr315 [23]. The preferential activation of these cascades in central memory T cells may therefore support the survival of these cells.

The OX40-dependent signaling either from APC or other activated CD4 T cells are reported to be required for the sufficient generation of memory CD4 T cells, at least under Th2 conditions [26,27]. GATA3 is required for the maintenance of Th2 cytokine production [28–30] and chromatin remodeling of the Th2 cytokine gene loci [28]. Memory Th2 cells maintain the Th2 features, such as selective Th2 cytokine production, a high-level expression of GATA3 mRNA and histone modifications of the Th2 cytokine gene loci in an IL-4-independent manner [13]. However, the molecules that control the maintenance of these Th2 features in memory Th2 cells have only recently been identified.

Table 1
Major components of the Polycomb/Trithorax complexes.

Drosophila protein	Protein domains	Mouse protein homologues
Polycomb group		
PC	Chromodomain	M33 (CBX2), CBX4, 6–8
PH	SAM	PHC1–3
PSC	RING	BMI1, Mel18
SCE	RING	RING1A/B
SCM	SAM, MBT, Zinc-finger	SCMH1, 2
E(Z)	SET	EZH1, 2
ESC/ESCL	WD40	EED
SU(Z)12	Zinc-finger	SUZ12
PHO/PHOL	Zinc-finger	YY1
ASX	PHD	ASXL1, 2
SFMBT	SAM, MBT	L3MBTL2, MBTD1
Trithorax group		
TRX	PHD, SET	MLL1, WBP7
ASH1	PHD, SET, BAH	ASH1
ASH2	PHD, SPRY	ASH2L
BRM	SNF2, Bromodomain	SMARCA4
MOR	SWIRM, SAINT	SMARCC1, 2
OSA	BRIGHT	ARID1B

Ref. [33].

4. PcG and TrxG gene products control cellular memory

In general, the gene expression in eukaryotic cells is maintained by epigenetic changes in the chromatin structure [31]. These changes include covalent posttranslational modification of nucleosomal histones, DNA methylation and remodeling of the nucleosome structure [32]. Recent studies provide substantial evidence indicating that PcG and TrxG proteins are key components for the maintenance of chromatin structures. The members of PcG and TrxG family proteins are summarized in Table 1 [33].

The PcG gene products were first identified in *Drosophila melanogaster* as molecules that are required for maintaining proper expression pattern of *homeotic* (*Hox*) genes [34]. The PcG gene is required for the maintenance of the repressed state of its target genes. PcG silencing involves at least two kinds of multimeric heterogenous protein complex, called Polycomb repressive complex 1 (PRC1) and PRC2 (Fig. 2A). The mammalian PRC1 complex has been isolated and identified from Hela cells [35]. The purified complex contains Ring1A/B, HPC1/2/3, HPH1/2, Bmi1 and Mel18. The PRC1 contains Polycomb (Pc) molecule, and the PRC1 is able to bind specifically to the tri-methylated histone H3-K27 via the chromodomain of the Pc molecules [36]. The key component of the mammalian PRC2 complex is the Enhancer of Zeste homolog (Ezh2) having an intrinsic histone methyltransferase activity for histone

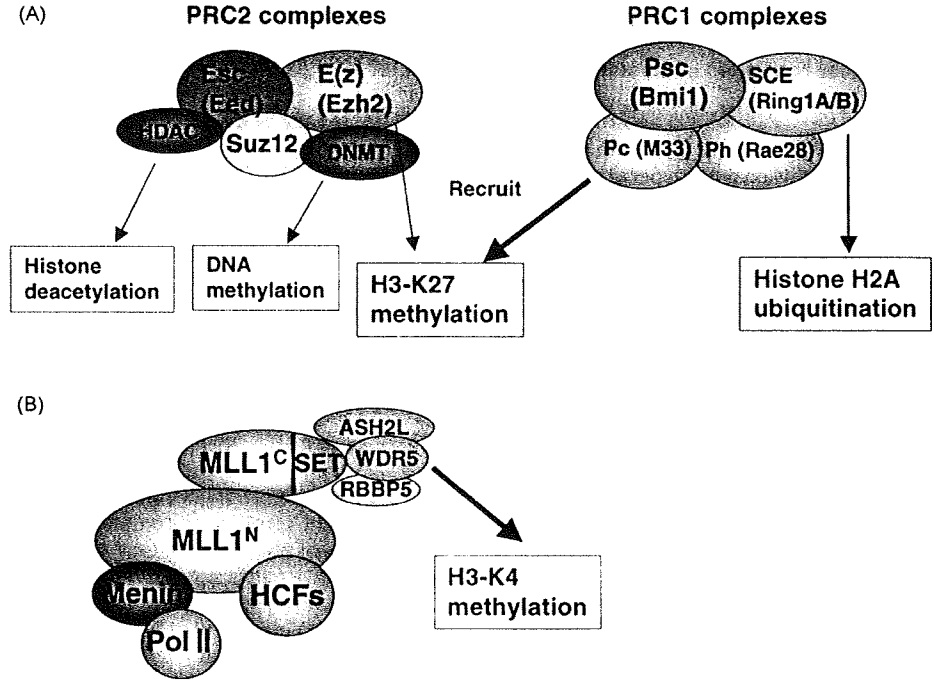

Fig. 2. Schematic representation of PcG and TrxG molecule complexes. (A) PcG proteins form at least two types of multiprotein complexes Polycomb repressive complex 1 (PRC1) and PRC2. E(z) has an intrinsic activity of tri-methylation of histone H3-K27 [51–54]. SCE (Ring 1B) monoubiquitinates histone H2A-K119 [55,56]. The chromodomain of Pc recognizes the tri-methylated histone H3-K27 [36]. PRC2 contains DNMTs (DNA methyl transferases) and HDACs (histone deacetylases). (B) A proposed Trx complex containing MLL1. MLL1 is reported to be associated with ASH2L, RbBP5, WDR5, and Menin [57]. RbBP5 and WDR5 are the WD40 repeat containing protein. The MLL complex has an activity of tri-methylation of histone H3-K4.

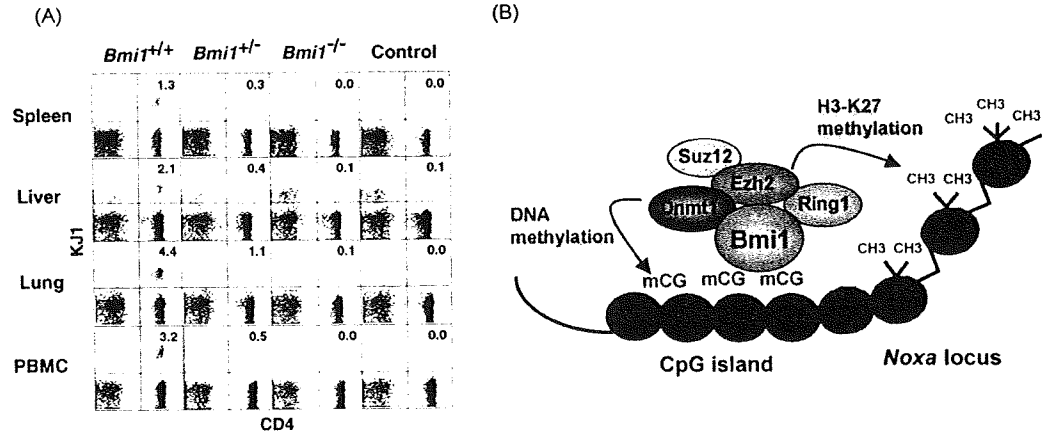

Fig. 3. Bmi1 regulates memory Th2 cell generation via direct binding to the *Noxa* gene. (A) Impaired generation of memory Th2 cells from *Bmi1*$^{-/-}$ effector Th2 cells. *Bmi1*$^{+/+}$, *Bmi1*$^{+/-}$ or *Bmi1*$^{-/-}$ effector Th2 cells with DO11.10 Tg background were intravenously transferred into syngeneic BALB/c mice. Five weeks later, the number of donor-derived KJ1^{+} memory Th2 cells was determined by flow cytometry. (B) A proposed Bmi1 containing PcG complex that regulates directly the expression of *Noxa*. Dnmt1, Ring1, Ezh2, and Suz12 are recruited to the CpG island of the *Noxa* gene locus, and the methylation of genomic DNA and histone H3-K27 of this region was detected [47].

H3-K27. Interestingly, DNA methyltransferase 1 (DNMT1) and histone deacetylases (HDACs) are also components of PRC2, and thus PRC2 appears to be able to negatively regulate the expression of target genes. The mammalian PRC2 complex contains at least two other PcG molecules, namely Suz12 and Eed.

The major role of TrxG gene products in the regulation of the *Hox* gene expression is to prevent PcG gene product-mediated silencing [37,38]. The mammalian homolog of *Drosophila trithorax* (*trx*) gene, *Mixed-Lineage Leukemia 1* (*MLL1*) gene is isolated as a common target of chromosomal translocations observed in human acute leukemias [39–43]. MLL1 forms a multi-component complex containing other TrxG protein, ASH2L (Fig. 2B), and has a histone methyltransferase activity specific for histone H3-K4, a modification typically associated with the transcriptionally active regions of chromatin [44,45].

5. Essential role of Bmi1 in the regulation of memory T cell survival

Memory T cells have several features associated with stem cells, and the similarity of the gene expression pattern between memory T/B cells and long-term hematopoietic stem cells is reported [46]. Similar to hematopoietic stem cells, memory T cells appear to possess the ability to proliferate in response to homeostatic signals. The PcG gene *Bmi1* has recently been implicated in the maintenance of hematopoietic [8,9], neural [10] and cancer stem cells [11]. The role of Bmi1 in the generation and maintenance of memory Th1/Th2 cells was investigated using the memory Th cell generation system stated above [47]. A *Bmi1* gene dose-dependent decrease in the numbers of memory Th2 cells is observed in all tissues tested (spleen, liver, lung, and peripheral blood mononuclear cells; PBMC) (Fig. 3A). Memory Th1 cell generation from *Bmi1*$^{-/-}$ effector Th1 cells is also impaired. This is also observed when they were transferred to lymphopenic BALB/c *nu/nu* mice [47]. These results clearly indicate that Bmi1 expression is required for the generation of memory Th1/Th2 cells.

Bmi1 has been reported to control cell proliferation and stem cell self-renewal by repressing the *Ink4a/Arf* locus [48]. This locus codes for two proteins, p16^{ink4a} and p19^{arf} (Ink4a and Arf), through the use of alternative reading frames. Ink4a is a cyclin D-dependent kinase inhibitor that induces cell cycle arrest following Rb activation. Arf induces p53 activation and p53-mediated cell death [49]. Although the increased expression of mRNA is observed in the *Ink4a/Arf* in *Bmi1*$^{-/-}$ memory Th2 cells, the deletion of the *Ink4a* and *Arf* genes failed to restore memory Th2 cell generation in *Bmi1*$^{-/-}$ [47]. Among the pro-apoptotic genes (*Bax*, *Puma*, *Noxa*, *Bim*, *Bad*, *Fas* and *Fas ligand*) that increased in *Bmi1*$^{-/-}$ Th2 cells, the level of *Noxa* mRNA remained high in *Bmi1*$^{-/-}$/*Ink4a*$^{-/-}$/*Arf*$^{-/-}$ Th2 cells. Noxa is a member of BH3-only protein family that initiates programmed cell death in various cells including lymphocytes. Bmi1 binds to the CpG islands at *Noxa* gene and suppresses the expression of *Noxa* through the maintenance of H3-K27 histone methylation. The other PcG family proteins, such as Ring1B and Suz12 also bind to the Noxa gene locus (Fig. 3B). In addition, Bmi1-dependent recruitment of Dnmt1 at the *Noxa* gene locus is observed. Furthermore, Noxa-deletion significantly rescued the effects of Bmi1-deficiency on memory Th2 cell generation. Mel-18, Mph1/Rae28, Ring1B and M33 are also found to be involved in the regulation of memory Th2 cell generation (unpublished observation). Therefore, Bmi1 containing PcG complex regulates memory Th cell survival at least in part through the regulation of *Noxa* gene expression.

6. Maintenance of memory Th2 cell function regulated by TrxG molecule, MLL1

Some of the effector Th cells are maintained as memory Th cells for a long time *in vivo* [4]. In memory Th2 cells, GATA3 is required for the maintenance of Th2 cytokine production [28–30] and chromatin remodeling of the Th2 cytokine gene loci [28]. Memory Th2 cells maintain the Th2 features, such as selective Th2 cytokine production upon recall stimulation, high-level expression of GATA3 mRNA and histone modifications of the Th2 cytokine gene loci in an IL-4-independent manner [13]. To maintain a high-level expression of GATA3 and subsequent Th2 cytokine production, MLL1, a member of TrxG protein, has been found to play a crucial role [50]. MLL is involved in one of the nuclear regulatory mechanism that establishes an epigenetic transcriptional memory system [12]. MLL1 forms a multi-component complex and mediates its epigenetic transcriptional effector functions via the SET domain-dependent histone methyltransferase activity [44,45] (Fig. 2B). MLL1 specifically methylates lysine 4 (K4) present on histone H3, a modification typically associated with the transcriptionally active regions of chromatin. A substantial decrease in the transcription of GATA3 accompanied by decreased methylation levels of H3-K4 at the GATA3 gene locus are detected in MLL1-knockdown Th2 cell lines and in *in vivo* generated memory Th2 cells with an *MLL1* heterozygous background [50]. MLL1 appears to regulate the memory Th2 responses through the control of chromatin remodeling at the

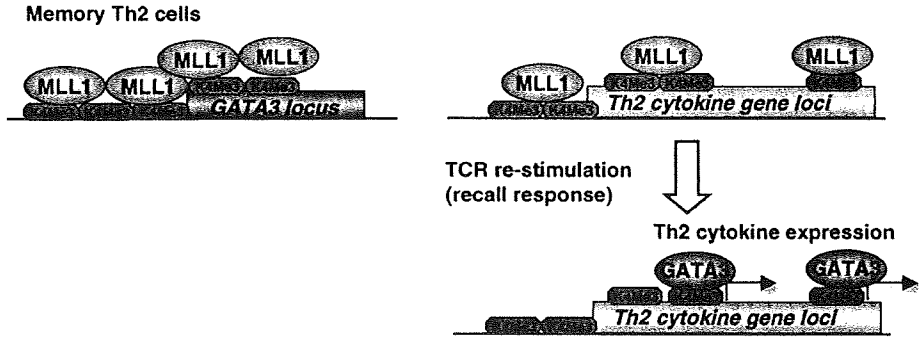

Fig. 4. MLL controls the maintenance of open chromatin of the GATA3 and Th2 cytokine gene loci in memory Th2 cells. In memory Th2 cells, MLL binds to the GATA3 gene locus and Th2 cytokine gene loci to maintain the open chromatin of these loci accompanied with histone H3-K4 methylation. Upon TCR restimulation during recall responses, GATA3 plays an important role in the transcription of Th2 cytokine genes. In memory Th2 cells, GATA3 does not play a major role in the regulation of chromatin status, but still plays an important role as a transcription factor for the Th2 cytokine gene.

GATA3 locus and subsequent GATA3 expression in Th2 cells (Fig. 4). In addition to the GATA3 gene locus, MLL1 is also found to be involved in the maintenance of activated chromatin status at the Th2 cytokine gene loci (Fig. 4). The direct binding of MLL1 at specific regions on the IL-4 and IL-13 gene loci as well as GATA3 gene locus is detected. Th2 cytokine production was severely reduced in *MLL1*-knockdown Th2 cell lines and *MLL1* heterozygous memory Th2 cells accompanied by decreased levels of H3-K9/14 acetylation and H3-K4 methylation. The antigen-induced allergic airway inflammatory responses *in vivo* are also compromised in *MLL1*$^{+/-}$ memory Th2 mice, thus suggesting a physiological role for MLL1 in the regulation of allergic reactions. Therefore, the maintenance of transcriptional expression of GATA3 in established Th2 cells including memory Th2 cells is epigenetically regulated by TrxG molecule MLL1.

7. Concluding remarks

In the past a few years, new insights into the molecular requirement for the epigenetic regulation of the function of CD4 T cells have emerged. PcG and TrxG gene products counteract each other in the epigenetic function during the development in *Drosophila* and mammals. In contrast, PcG and TrxG gene products have been found to regulate different processes during the generation and the maintenance of memory CD4 T cells. The mode of regulation by PcG and TrxG is also distinct. PcG and TrxG complexes are heterogenous and the components in the complexes appear to be different in different cell types and also in the different developmental stages. Therefore, the analyses of the precise molecular actions of a particular complex formed in a specific process during the initiation and the maintenance of functional memory Th1/Th2 cell would be most important. Moreover, similar to Th1/Th2 cells, further investigation of the regulation by RcG and TrxG molecules in other functionally distinct CD4 T cell subsets, such as Th17, follicular helper T (Tfh), regulatory T (Treg) and Th9 would also be intriguing.

Acknowledgements

This work was supported by Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT (Japan), and by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid: for Scientific Research on Priority Areas #17016010, #20060003; Scientific Research (B) #17390139, Scientific Research (C) #20590485, Exploratory Research #19659121: Special Coordination Funds for Promoting Science and Technology, and: Cancer Translational Research Project), the Ministry of Health, Labor and Welfare (Japan).

References

[1] Marrack P, Kappler J. Control of T cell viability. Annu Rev Immunol 2004;22:765–87.
[2] Stockinger B, Kassiotis G, Bourgeois C. CD4 T-cell memory. Semin Immunol 2004;16:295–303.
[3] Dutton RW, Bradley LM, Swain SL. T cell memory. Annu Rev Immunol 1998;16:201–23.
[4] Sprent J, Surh CD. T cell memory. Annu Rev Immunol 2002;20:551–79.
[5] Nakayama T, Yamashita M. Initiation and maintenance of Th2 cell identity. Curr Opin Immunol 2008;20:265–71.
[6] Satijn DP, Otte AP. Polycomb group protein complexes: do different complexes regulate distinct target genes? Biochim Biophys Acta 1999;1447:1–16.
[7] van Lohuizen M. The trithorax-group and polycomb-group chromatin modifiers: implications for disease. Curr Opin Genet Dev 1999;9:355–61.
[8] Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003;423:302–5.
[9] Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity 2004;21:843–51.
[10] Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. *Bmi-1* dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 2003;425:962–7.
[11] Lessard J, Sauvageau G. *Bmi-1* determines the proliferative capacity of normal and leukaemic stem cells. Nature 2003;423:255–60.
[12] Francis NJ, Kingston RE. Mechanisms of transcriptional memory. Nat Rev Mol Cell Biol 2001;2:409–21.
[13] Yamashita M, Shinnakasu R, Nigo Y, Kimura M, Hasegawa A, Taniguchi M, et al. Interleukin (IL)-4-independent maintenance of histone modification of the IL-4 gene loci in memory Th2 cells. J Biol Chem 2004;279:39454–64.
[14] Rogers PR, Dubey C, Swain SL. Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. J Immunol 2000;164:2338–46.
[15] Jameson SC. Maintaining the norm: T-cell homeostasis. Nat Rev Immunol 2002;2:547–56.
[16] Schluns KS, Lefrancois L. Cytokine control of memory T-cell development and survival. Nat Rev Immunol 2003;3:269–79.
[17] Lantz O, Grandjean I, Matzinger P, Di Santo JP. γ Chain required for naive CD4$^+$ T cell survival but not for antigen proliferation. Nat Immunol 2000;1:54–8.
[18] Seddon B, Tomlinson P, Zamoyska R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. Nat Immunol 2003;4:680–6.
[19] Kondrack RM, Harbertson J, Tan JT, McBreen ME, Surh CD, Bradley LM. Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med 2003;198:1797–806.
[20] Li J, Huston G, Swain SL. IL-7 promotes the transition of CD4 effectors to persistent memory cells. J Exp Med 2003;198:1807–15.
[21] Paukku K, Silvennoinen O. STATs as critical mediators of signal transduction and transcription: lessons learned from STAT5. Cytokine Growth Factor Rev 2004;15:435–55.
[22] White E. The pims and outs of survival signaling: role for the Pim-2 protein kinase in the suppression of apoptosis by cytokines. Genes Dev 2003;17:1813–6.
[23] Riou C, Yassine-Diab B, Van grevenynghe J, Somogyi R, Greller LD, Gagnon D, et al. Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of CD4+ central memory T cells. J Exp Med 2007;204:79–91.
[24] Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905–27.
[25] Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 2004;117:225–37.

[26] Soroosh P, Ine S, Sugamura K, Ishii N. OX40–OX40 ligand interaction through T cell–T cell contact contributes to CD4 T cell longevity. J Immunol 2006;176:5975–87.
[27] Weinberg AD, Evans DE, Thalhofer C, Shi T, Prell RA. The generation of T cell memory: a review describing the molecular and cellular events following OX40 (CD134) engagement. J Leukoc Biol 2004;75:962–72.
[28] Yamashita M, Ukai-Tadenuma M, Miyamoto T, Sugaya K, Hosokawa H, Hasegawa A, et al. Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. J Biol Chem 2004;279:26983–90.
[29] Pai SY, Truitt ML, Ho IC. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. Proc Natl Acad Sci USA 2004;101:1993–8.
[30] Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, et al. Conditional deletion of *Gata3* shows its essential function in T_H1–T_H2 responses. Nat Immunol 2004;5:1157–65.
[31] Horn PJ, Peterson CL. Molecular biology. Chromatin higher order folding—wrapping up transcription. Science 2002;297:1824–7.
[32] Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074–80.
[33] Schwartz YB, Pirrotta V. Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet 2007;8:9–22.
[34] Lewis EB. A gene complex controlling segmentation in *Drosophila*. Nature 1978;276:565–70.
[35] Levine SS, Weiss A, Erdjument-Bromage H, Shao Z, Tempst P, Kingston RE. The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol Cell Biol 2002;22:6070–8.
[36] Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, Khorasanizadeh S. Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev 2003;17:1870–81.
[37] Poux S, Horard B, Sigrist CJ, Pirrotta V. The *Drosophila trithorax* protein is a coactivator required to prevent re-establishment of polycomb silencing. Development 2002;129:2483–93.
[38] Klymenko T, Muller J. The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. EMBO Rep 2004;5:373–7.
[39] Ziemin-van der Poel S, McCabe NR, Gill HJ, Espinosa III R, Patel Y, Harden A, et al. Identification of a gene, *MLL*, that spans the breakpoint in 11q23 translocations associated with human leukemias. Proc Natl Acad Sci USA 1991;88:10735–9.
[40] Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimino G, et al. The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to *Drosophila trithorax*, to the AF-4 gene. Cell 1992;71:701–8.
[41] Tkachuk DC, Kohler S, Cleary ML. Involvement of a homolog of *Drosophila trithorax* by 11q23 chromosomal translocations in acute leukemias. Cell 1992;71:691–700.
[42] Thirman MJ, Gill HJ, Burnett RC, Mbangkollo D, McCabe NR, Kobayashi H, et al. Rearrangement of the MLL gene in acute lymphoblastic and acute myeloid leukemias with 11q23 chromosomal translocations. N Engl J Med 1993;329:909–14.
[43] Popovic R, Zeleznik-Le NJ. MLL: how complex does it get? J Cell Biochem 2005;95:234–42.
[44] Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, et al. MLL targets SET domain methyltransferase activity to *Hox* gene promoters. Mol Cell 2002;10:1107–17.
[45] Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, Wassell R, et al. ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell 2002;10:1119–28.
[46] Luckey CJ, Bhattacharya D, Goldrath AW, Weissman IL, Benoist C, Mathis D. Memory T and memory B cells share a transcriptional program of self-renewal with long-term hematopoietic stem cells. Proc Natl Acad Sci USA 2006;103:3304–9.
[47] Yamashita M, Kuwahara M, Suzuki A, Hirahara K, Shinnaksu R, Hosokawa H, et al. Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. J Exp Med 2008;205:1109–20.
[48] Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. Nature 1999;397:164–8.
[49] Lowe SW, Sherr CJ. Tumor suppression by *Ink4a–Arf*: progress and puzzles. Curr Opin Genet Dev 2003;13:77–83.
[50] Yamashita M, Hirahara K, Shinnakasu R, Hosokawa H, Norikane S, Kimura MY, et al. Crucial role of MLL for the maintenance of memory T helper type 2 cell responses. Immunity 2006;24:611–22.
[51] Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, Pirrotta V. *Drosophila* enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 2002;111:185–96.
[52] Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, et al. Histone methyltransferase activity of a *Drosophila* Polycomb group repressor complex. Cell 2002;111:197–208.
[53] Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002;298:1039–43.
[54] Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 2002;16:2893–905.
[55] Wang H, Wang L, Erdjument-Bromage H, Vidal M, Tempst P, Jones RS, et al. Role of histone H2A ubiquitination in Polycomb silencing. Nature 2004;431:873–8.
[56] de Napoles M, Mermoud JE, Wakao R, Tang YA, Endoh M, Appanah R, et al. Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell 2004;7:663–76.
[57] Yokoyama A, Wang Z, Wysocka J, Sanyal M, Aufiero DJ, Kitabayashi I, et al. Leukemia proto-oncoprotein MLL forms a SET1-like histone methyltransferase complex with menin to regulate *Hox* gene expression. Mol Cell Biol 2004;24:5639–49.

Available online at www.sciencedirect.com

ScienceDirect

Cancer Letters 262 (2008) 239–247

CANCER Letters

www.elsevier.com/locate/canlet

Mutagenicity of 8-nitroguanosine, a product of nitrative nucleoside modification by reactive nitrogen oxides, in mammalian cells

Kazuyoshi Kaneko [a,b,1], Teruo Akuta [a,1], Tomohiro Sawa [a], Ha Won Kim [c,2], Shigemoto Fujii [a], Tatsuya Okamoto [a], Hitoshi Nakayama [b], Hajime Ohigashi [c], Akira Murakami [c], Takaaki Akaike [a,*]

[a] *Department of Microbiology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan*
[b] *Department of Molecular Cell Function, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Ohe-Honmachi, Kumamoto 862-0973, Japan*
[c] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*

Received 18 June 2007; received in revised form 30 November 2007; accepted 3 December 2007

Abstract

8-Nitroguanosine is a nitratively modified nucleoside that is formed endogeneously under inflammatory conditions dependent on nitric oxide production, particularly associated with cancer risks. Here, we investigated the mutagenic potential of 8-nitroguanosine in mammalian cells. Treatment with 8-nitroguanosine (10–1000 μM) for 1 h significantly increased (by 6–8 times) the mutation frequency of the xanthine–guanine phosphoribosyltransferase (*gpt*) gene in AS52 cells without cytotoxic effects. 8-Nitroguanosine treatment induced a G-to-T transversion in *gpt* gene at position 86. It also significantly increased levels of abasic sites in DNA. These observations suggest that formation of 8-nitroguanosine may contribute to the pathogenesis of inflammation-associated carcinogenesis.

Keywords: Nitric oxide; Inflammation; 8-Nitroguanosine; Mutation; Abasic site

Abbreviations: NOS, nitric oxide synthase; iNOS, inducible form of NOS; TG^r, 6-thioguanine-resistant; FBS, fetal bovine serum; MPA, mycophenolic acid; HBSS, Hank's balanced salt solution; DMSO, dimethyl sulfoxide; RT-PCR, reverse transcriptase-polymerase chain reaction; G3PDH, glyceraldehydes-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; APE1, AP endonuclease 1.

* Corresponding author. Tel.: +81 96 373 5100; fax: +81 96 362 8362.
E-mail address: takakaik@gpo.kumamoto-u.ac.jp (T. Akaike).

[1] These authors contributed equally to this work.

[2] Present address: Department of Pharmacology, The University of Illinois at Chicago, 909 South Wolcott Avenue, Chicago, IL 60612, USA.

0304-3835/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2007.12.007

1. Introduction

Chronic inflammation caused by microbial, chemical, or physical agents has been associated with increased risk of human cancer in various tissues and organs. For example, inflammation-induced carcinogenesis is well documented for *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis in gastric cancer, inflammatory bowel disease (ulcerative colitis and Crohn's disease) in colorectal cancer, chronic viral hepatitis in liver cancer, liver fluke infestation in cholangiocarcinoma, and Barrett's esophagus in esophageal cancer [1–3]. Activated macrophages and lymphocytes infiltrate sites of inflammation and produce and/or stimulate stromal cells to produce cytokines, growth factors, and angiogenic factors. They also produce large amounts of reactive oxygen and nitrogen oxides that can react with nucleic acids, proteins, and cell membranes to induce oxidation, nitration, and nitrosation modifications [4,5]. These modifications affect biochemical functions of the molecules. If these modifications are not repaired or metabolized appropriately, cells undergo mutagenesis or apoptosis, the second of which may be implicated in activation of tissue regeneration [4].

8-Nitroguanine is a nitratively modified nucleic acid that is formed endogeneously under inflammatory conditions dependent on nitric oxide (NO) production [6–9]. Accumulating evidence has suggested the enhanced formation of 8-nitroguanine-related compounds in various pathological conditions associated with cancer risk. For instance, we recently reported intense immunostaining for 8-nitroguanosine in metaplastic regenerated epithelial cells from patients with idiopathic pulmonary fibrosis, who show a high incidence of lung cancer [10]. 8-Nitroguanosine immunostaining colocalized with the inducible form of NO synthase (iNOS) [10]. Positive immunostaining was also evident in malignant epithelial cells in specimens of squamous cell carcinoma from patients with lung cancer [10]. Cigarette smoking was reportedly associated with increased urinary excretion of 8-nitroguanine [11]. Also, enhanced formation of 8-nitroguanine derivatives and their colocalization with iNOS were observed in various specimens of human tissues including gastric gland epithelium of gastritis patients with *H. pylori* infection [12], intrahepatic cholangiocarcinoma [13], and liver from patients with chronic hepatitis C [14].

8-Nitroguanine formed in DNA has been considered as mutagenic lesion because of its rapid removal from DNA strand by depurination to form mutagenic abasic sites [15], that can possibly induce G-to-T transversions. Recently, in a primer extension reaction with a combination of an oligodeoxynucleotide containing a single 8-nitroguanine residue and mammalian DNA polymerase (pol α, β, η, or κΔC), 8-nitroguanine directly promoted G-to-T transversions without depurination [16]. However, whether 8-nitroguanine in DNA can indeed induce mutation in cells remains to be proven experimentally.

In contrast to the deoxyribonucleoside forms, 8-nitroguanine in ribonucleoside forms, such as 8-nitroguanosine (Fig. 1), resist depurination [17]. We previously demonstrated that 8-nitroguanosine was mutagenic for RNA viruses (e.g., Sendai virus) [18]. The type of mutation induced by 8-nitroguanosine was a C-to-U transition; G-to-U transversions were observed only rarely in viral RNA after 8-nitroguanosine treatment [18]. 8-Nitroguanosine also functions as a pro-oxidant and generates superoxide radicals in the presence of certain reductases such as NADPH-cytochrome P450 reductase and all isoforms of NOS [9,19], and it causes cellular oxidative stress [18]. Our immunohistochemical analyses showed that cytosol is the primary site of 8-nitroguanosine immunostaining [9,10]. This cytoplasmic localization of 8-nitroguanosine immunostaining supports the notion that 8-nitroguanosine-related compounds form in nucleotide pool and RNA.

The aims of this study were to investigate whether 8-nitroguanosine is mutagenic in mammalian cells and to identify the mutation pattern. We used AS52 cells, which were generated from parent Chinese hamster ovary cells. AS52 cells lack a normal X-linked mammalian hypoxanthine–guanine phosphoribosyltransferase (*hprt*) gene but contain

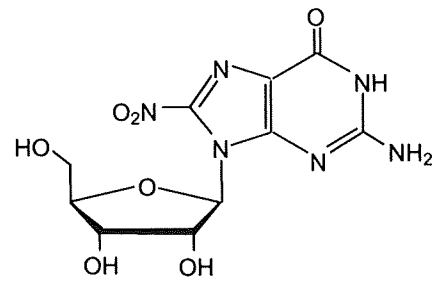

Fig. 1. Chemical structure of 8-nitroguanosine.

a single copy of the *Escherichia coli* xanthine–guanine phosphoribosyltransferase (*gpt*) gene that is functionally expressed via the SV40 early promoter and is stably integrated into the genome [20]. Mutation of the *gpt* gene in AS52 cells can readily be identified by phenotypic selection of 6-thioguanine resistant (TG^r) mutants. Therefore, we isolated 8-nitroguanosine-induced TG^r mutants and sequenced the mutated *gpt* gene so as to analyze the mutation pattern. We also examined 8-nitroguanosine-induced oxidative damage and formation of abasic sites in DNA in AS52 cells.

2. Materials and methods

2.1. Chemicals

8-Nitroguanosine was synthesized and purified according to reported procedures [9,19]. All other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) or Wako Pure Chemicals (Osaka, Japan) and used without further purification.

2.2. Cell culture

AS52 cells were kindly donated by Dr. Marshall V. Williams, The Ohio State University, Columbus, OH, USA. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere, in Ham's F-12 medium containing L-glutamate supplemented with 5% fetal bovine serum (FBS) and mycophenolic acid (MPA) additives (10 μg/mL MPA, 25 μg/mL adenine, 50 μM thymidine, 250 μg/mL xanthine, and 3 μM aminopterin).

2.3. Mutation assay

The AS52 mutation assay was performed as reported previously [20,21]. Briefly, on day −1, AS52 cells were preincubated in Ham's F-12 medium without MPA additives at a density of 10^6 cells/100-mm dish. On day 0, cells were washed three times with Hank's balanced salt solution (HBSS) and were then treated with H_2O_2 (100 μM) or 8-nitroguanosine (10–1000 μM) dissolved in FBS- and MPA-free medium for 1 h. Dimethyl sulfoxide (DMSO) was used as a vehicle control. After treatment, cells were washed three times with HBSS and were then incubated with Ham's F-12 medium without MPA additives. On days +3 and +6, cells were subcultured at a density of 10^6 cells/100-mm dish. On day +9, cells were cultured in 100-mm dishes at a density of 2×10^5 cells/dish in Ham's F-12 medium containing 5% FBS and 10 μM 6-thioguanine. Cells were incubated for another 10 days and were then examined for development of TG^r mutants. After cells were fixed with a solution containing methanol, acetic acid, and water (50:7:43), they were stained with a 1% crystal violet solution. Only colonies containing at least 50 cells were counted. Mutation frequency was expressed as number of mutants/10^6 clonable cells. Independent TG^r mutants were isolated for analysis of the gene mutation, described below.

Cytotoxicity was determined on day +4 and plating efficiency on day +7. Briefly, AS 52 cells were plated at a density of 200 cells/60-mm dish in Ham's F-12 medium lacking MPA additives, and incubated at 37 °C for 7 days (three to five dishes). Only those clones containing at least 50 cells/colony were counted.

2.4. Sequence analysis

For analysis of the *gpt* gene, total RNA extracted from each mutant by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) with the following oligonucleotide primers: sense (bases −199 to −172) 5′-AAGCTTGGACACAAGACAGGCTTGCGAG-3′ and antisense (bases 515 to 544) 5′-GCCTCCAGAAT ACTTACTGGAAACTATTG-3′ [22]. The 0.7-kb RT-PCR product was purified for DNA sequence analysis by using the QIAGEN gel extraction kit (QIAGEN GmbH, Hilden, Germany). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control; the forward primer was 5′-AAACCCATCACCAT CTTCCA-3′ and the reverse primer was 5′-CAGGG GTTTCTTACTCCTTG-3′. PCR products were sequenced via a dideoxy method with a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA, USA) with the primer sequence (bases −58 to −49) 5′-C GCAACCTATTTTCC-3′ and BigDye Terminator v1.1 Cycle Sequencing kit.

2.5. Immunocytochemical analysis of oxidative DNA damage

Oxidative DNA damage was determined immunocytochemically with anti-8-oxodeoxyguanosine antibody (Japan Institute for the Control of Aging, Fukuroi, Japan). After 1 h of treatment with 8-nitroguanosine or H_2O_2 as mentioned above, AS52 cells were washed three times with phosphate-buffered saline (PBS) and were then fixed with Zamboni solution (4% paraformaldehyde and 10 mM picric acid in 0.1 M phosphate buffer, pH 7.4) at 4 °C for 7 h. After three washes with PBS, cells were permeabilized with 0.5% Triton X-100 at room temperature for 15 min and were washed again with PBS. Nonspecific antigenic sites were blocked by incubation with BlockAce (Dainippon Pharmaceutical, Osaka, Japan) at 4 °C overnight. Cells were stained with 10 μg/mL monoclonal anti-8-oxodeoxyguanosine antibody at 4 °C overnight, washed in PBS, and probed with goat Cy3-conjugated anti-mouse antibody (Amersham Biosciences, Piscataway, NJ, USA) at room tem-

perature for 30 min. Cells were mounted under coverslips with SlowFade Gold antifade reagent (Invitrogen). Images were acquired by means of a fluorescence microscope (Nikon, Tokyo, Japan). NIH Image was used for densitometric analysis to quantitate the intensity of immunostaining. This intensity was measured for at least 10 cells, the results being expressed as averages ± SD.

2.6. Determination of abasic sites in genome DNA

The number of abasic sites in genome DNA was measured by using a commercially available biotin-containing aldehyde-reactive probe [23] (DNA Damage Quantification Kit; Dojindo Laboratories, Kumamoto, Japan). AS52 cells were treated with H_2O_2 or 8-nitroguanosine for 1 h as mentioned above and were then washed three times with PBS. Cells were then further incubated in Ham's F-12 medium containing 5% FCS for 5 or 8 h. Genomic DNA was isolated from cells by the QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instruction. Levels of biotin-tagged abasic sites in isolated DNA were determined colorimetrically by an enzyme-linked immunosorbent assay-like assay with an avidin–biotin complex conjugated to horseradish peroxidase as the indicator enzyme.

2.7. Statistical analysis

Student's *t*-test was used to evaluate statistical significance. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Mutagenicity of 8-nitroguanosine in AS52 cells in culture

Treatment with H_2O_2 (100 μM) or 8-nitroguanosine (10 – 1000 μM) for 1 h had no apparent cytotoxicity for AS52 cells (Fig. 2a). Measurement of the frequency of generation of TG^r mutants in DMSO-treated cells provided a spontaneous mutation frequency of $4.5 \pm 1.5/10^6$ cells. Treatment of AS52 cells with 100 μM H_2O_2, a mutagenic oxidant, increased the mutation frequency to $36.5 \pm 2.8/10^6$ cells, as shown in Fig. 2b. We found that 8-nitroguanosine treatment significantly increased the mutation frequency in AS52 cells in a concentration-dependent manner (Fig. 2b). 8-Nitroguanosine at 1000 μM induced a six-fold increase in the mutation frequency ($29.8 \pm 1.7/10^6$ cells), which was, like that induced by 100 μM H_2O_2, significantly different from the spontaneous mutation frequency. This result clearly suggests a mutagenic potential for 8-nitroguanosine in AS52 cells.

To determine the mutation pattern induced by 8-nitroguanosine, we performed RT-PCR followed by sequencing of *gpt* cDNA obtained from isolated TG^r cells. Fig. 3 shows that we obtained RT-PCR products of approximately 0.7 kbp from both spontaneous mutants and 8-nitroguanosine-treated TG^r AS52 cells, whereas we found no such PCR products in H_2O_2-treated TG^r AS52 cells. H_2O_2 treatment may have induced the mutation so that the promoter region of the *gpt* gene lost its transcriptional capability, or an abnormal transcript that could not be detected by PCR, as used in this study, was produced. We confirmed the lack of mutations in RT-

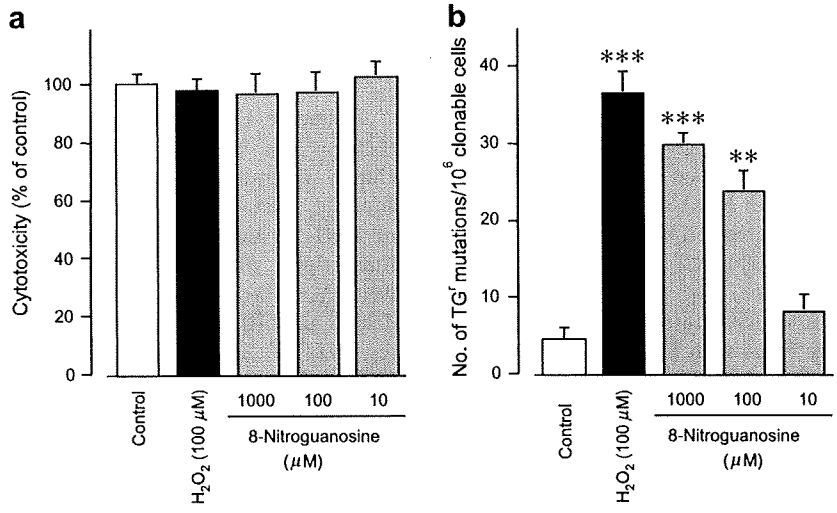

Fig. 2. Cytotoxicity (a) and mutagenic activity (b) of H_2O_2 and 8-nitroguanosine in AS52 cells. AS52 cells were treated with MPA-free medium containing 1% DMSO (vehicle control), 100 μM H_2O_2 (positive control), or concentrations of 10–1000 μM 8-nitroguanosine for 1 h. The mutation frequency was determined by measuring the numbers of TG^r mutants. Data are shown as means ± SD ($n = 3$). $^{**}P < 0.001$ and $^{***}P < 0.0001$ compared with vehicle controls.

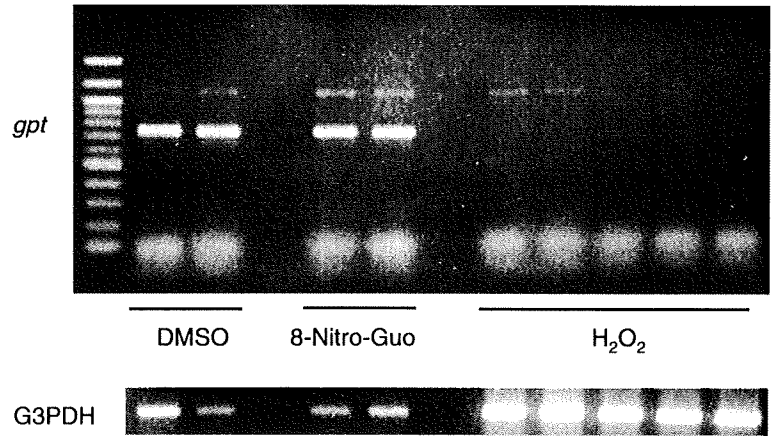

Fig. 3. RT-PCR amplification of the *gpt* gene from parental AS52 cells, spontaneous mutant TG^r cells, and TG^r cells generated by treatment with H_2O_2 or 8-nitroguanosine. Total RNA extracted from each mutant was subjected to RT-PCR amplification for the *gpt* gene as described in Section 2.

Table 1
Mutation patterns of the *gpt* gene in TG^r mutants and predicted amino acid changes

Mutant	Position	Sequence alteration	Target sequence	Predicted amino acid change
DMSO ($n = 10$)	373–374	2-bp deletion	GAC TAT GTT	Frameshift
8-Nitroguanosine ($n = 15$)	86	G:C to T:A	CAA TGG AAA	29 Trp to Leu

PCR products obtained from parental AS52 cells (data not shown). Sequence analysis indicated that the spontaneous mutation occurred at position 373–374, with a 2-bp deletion, possibly leading to a frameshift mutation (Table 1). In contrast, all TG^r mutants induced by 8-nitroguanosine treatment showed a single point mutation at position 86 with G-to-T transversions. This mutation results in the substitution of the amino acid tryptophan with leucine (Table 1). We found no large deletions, insertions, or tandem or multiple mutations in the *gpt* gene in both spontaneous mutants and 8-nitroguanosine-induced TG^r AS52 cells.

3.2. Occurrence of oxidative DNA damage in AS52 cells treated with H_2O_2 or 8-nitroguanosine

We examined the formation of 8-oxodeoxyguanosine, a marker of oxidative DNA damage and mutagenic lesions [24], in cells to determine whether 8-nitroguanosine treatment would induce oxidative DNA damage in AS52 cells, as expected from its pro-oxidant activity. Fig. 4 shows that H_2O_2 treatment significantly increased immunoreactivity for 8-oxodeoxyguanosine, which suggests induction of oxidative DNA damage. In contrast, 8-oxodeoxyguanosine staining increased only slightly in cells treated with 8-nitroguanosine compared with the control. Thus, 8-nitroguanosine treatment did not induce significant oxidative DNA damage in AS52 cells under current conditions.

3.3. 8-Nitroguanosine-enhanced formation of abasic sites in AS52 cells

Abasic sites in DNA are mutagenic lesions that induce X-to-T substitutions, where X represents an abasic site [25]. We investigated whether 8-nitroguanosine treatment could modulate genome levels of abasic sites in AS52 cells. As shown in Table 2, H_2O_2 treatment moderately increased formation of abasic sites in AS52 cells. However, it is noteworthy that 8-nitroguanosine treatment induced formation of abasic sites in AS52 cells to a greater degree than did H_2O_2 treatment.

4. Discussion

iNOS-dependent overproduction of NO and subsequent conversion to reactive nitrogen oxides such as peroxynitrite and nitrogen dioxide can cause cellular stress by inducing nitrative modifications of DNA, RNA, nucleosides and nucleotides, proteins, and lipids [4–8]. As mentioned earlier, nitrative guanine modification is strongly enhanced in various cancer-associated diseases. However, how this modification contributes to mutagenesis and carcinogenesis has remained unclear. In this study, we demonstrated for the first time that 8-nitroguanosine is a potential mutagen in AS52 cells. Sequence

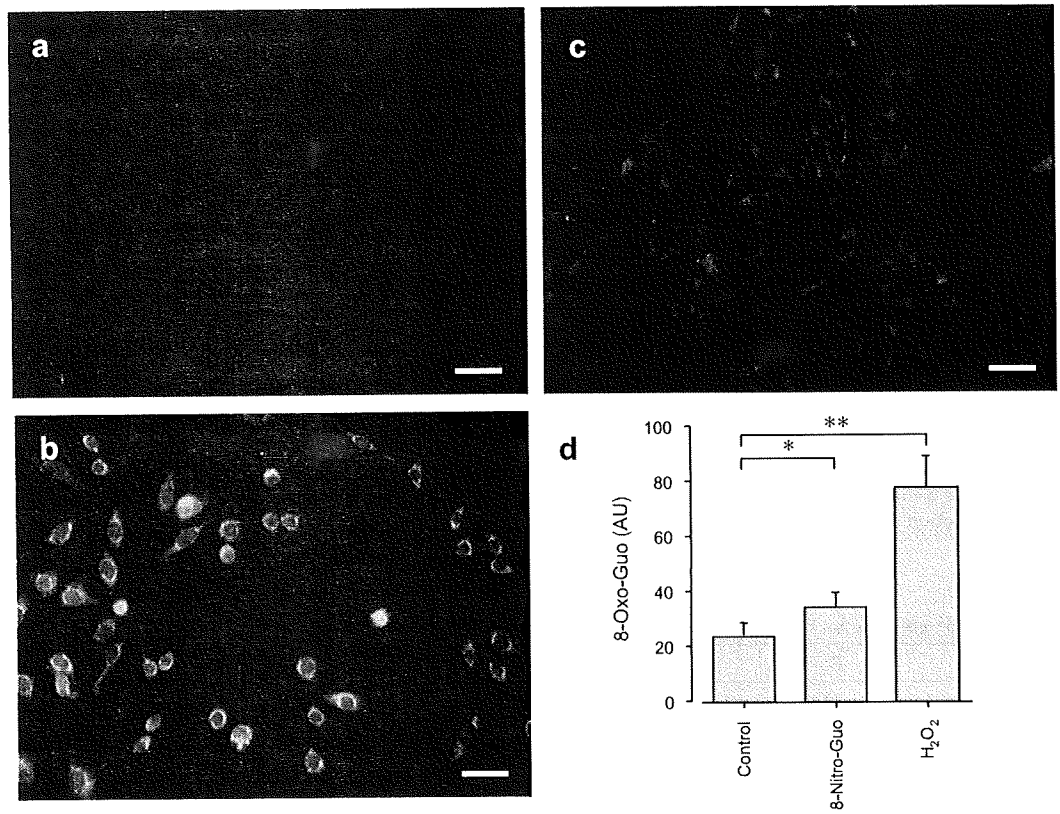

Fig. 4. Immunocytochemical analysis of oxidative DNA damage in AS52 cells. Cell levels of 8-oxodeoxyguanosine, as an indication of oxidative DNA damage, were determined by using mouse monoclonal anti-8-oxodeoxyguansoine antibody. AS52 cells were treated for 1 h with MPA-free medium containing 1% DMSO (a), 100 μM H_2O_2 (b), or 100 μM 8-nitroguanosine (c). Cells were then fixed and stained with anti-8-oxodeoxyguanosine antibody. Scale bars, 20 μm (a–c). (d) The intensity of immunostaining was determined as described in Section 2. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with controls.

Table 2
8-Nitroguanosine-dependent increase in abasic sites in AS52 cells

Time after treatment	Number of abasic sites/10^5 nucleotides		
	Control	Treatment with H_2O_2 (100 μM)	Treatment with 8-nitroguanosine (100 μM)
5 h	0.92 ± 0.16	1.80 ± 0.09*	2.53 ± 0.09**,***
8 h	0.18 ± 0.16	1.48 ± 0.07*	2.51 ± 0.30**,***

Data are shown as means ± SD ($n = 3$).
$^{*}P < 0.002$ and $^{**}P < 0.001$ compared with controls; $^{***}P < 0.005$ compared with H_2O_2 treatment.

analysis revealed that 8-nitroguanosine treatment induced only G-to-T transversions at position 86 in the *gpt* gene. This single point mutation caused substitution of the amino acid leucine for tryptophan, which led to functional impairment of gpt. This mutation pattern was different from that found in spontaneous TG^r mutants, which showed mainly a 2-bp deletion at position 373–374. Our data thus demonstrate that formation of a low-molecular-weight nitrated product of guanosine can be a potential source for induction of mutation in mammalian cells.

G-to-T transversions are common somatic mutations associated with human cancer [26,27]. This type of mutation may be caused by oxidative modification of guanine, which produces, for example, 8-oxodeoxyguanosine [28] and its further oxidized products including spiroiminodihydantoin, guanidinohydantoin, and iminoallantoin [29]. As mentioned earlier, 8-nitroguanosine can generate

superoxide radical in the presence of certain reductases such as cytochrome P450 reductase and NOSs [9,19]. It was thus hypothesized that 8-nitroguanosine might produce oxidative DNA damage via formation of oxygen radicals in cells. We used 8-oxodeoxyguanosine as a biomarker for oxidative DNA damage [24]. As shown in Fig. 4, 8-oxodeoxyguanosine formation was significantly increased by treatment with H_2O_2. Our data, however, failed to support the involvement of oxidative DNA damage in increased G-to-T transversions mediated by 8-nitroguanosine treatment, because we found only moderately increased 8-oxodeoxyguanosine values in 8-nitroguanosine-treated AS52 cells (Fig. 4).

We could detect basal level of abasic sites in control AS52 cells in a range of $0.18 - 0.92/10^5$ nucleotides (Table 2). Such level was consistent with that reported previously [23]. Abasic sites are known to be mutagenic yielding mainly single base substitutions [25]. However, no apparent single base substitution was detected in spontaneous TG^r mutants (Table 1). This suggests that such basal level abasic sites may not contribute to the significant extent for generation of TG^r mutants under current experimental setting. On the other hand, we found that 8-nitroguanosine-induced TG^r mutants possess single base substitutions as a major mutation type (Table 1). Recent study reported that abasic sites are not distributed randomly in DNA, but rather form clusters in a small region [30]. Such an uneven distribution of abasic sites has been considered to imply in induction of inefficient repair, leading to the mutation. In this context, abasic sites induced by 8-nitroguanosine treatment may be localized nonrandomly in gpt locus, promoting the mutagenic effects of abasic sites for the generation of TG^r mutants.

One possible mechanism by which 8-nitroguanosine can induce abasic site formation is that 8-nitroguanosine is incorporated in DNA to form labile 8-nitroguanine-containing sites, which are then depurinated from DNA strand to form abasic sites. For this mechanism, 8-nitroguanosine should be metabolized to 8-nitro-2'-deoxyguanosine triphosphate (8-nitro-dGTP), a precursor of DNA synthesis. In cells, guanosine can be metabolized to dGTP by salvage pathway [31,32]. In salvage pathway, guanosine is first metabolized to guanine by purine nucleoside phosphorylase. Guanine is then converted to GMP by hypoxanthine–guanine phosphoribosyl-transferase. By the action of guanylate kinase, GMP is phosphorylated to form GDP, which is subsequently converted to dGDP by ribonucleotide reductase. Finally, dGTP is formed from dGDP by nucleoside diphosphate kinase. Further studies are required to clarify whether 8-nitroguanosine can be incorporated in DNA via salvage pathway-dependent mechanism.

Abasic sites formed can be repaired by AP endonuclease 1 (APE1), which is responsible for more than 95% of cellular abasic site incision activity [33,34]. APE1 cleaves phosphodiester bonds hydrolytically in an Mg^{2+}-dependent manner, which leaves a 3′-hydroxyl group and a 5′-deoxyribose phosphate group flanking the nucleotide gap. Further repair can be accomplished via two pathways that involve different subsets of enzymes and that result in replacement of one (short-patch pathway) or more (long-patch pathway) nucleotides.

We recently identified formation of a new nitrated cyclic nucleotide, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP), in activated macrophages dependent on NO overproduction [35]. Quantitative analyses indicated that the intracellular levels of 8-nitro-cGMP (ca. 70 – 550 nM; dependent on cellular glutathione level) was several folds higher than those of 8-nitroguanine and 8-nitroxanthine [35]. It is thus important to elucidate how this nitrated nucleotide affect mutagenesis and tumorigenesis process, and further study is currently ongoing.

Agents that reduce nitrative stress may possess chemopreventive activity for inflammation-associated carcinogenesis [36]. These agents include (i) antagonists of pro-inflammatory cytokines, (ii) inhibitors of signaling pathways for iNOS expression (e.g., nuclear factor-κB), (iii) inhibitors of iNOS, and (iv) scavengers of reactive nitrogen oxides [36]. Indeed, certain naturally occurring chemopreventive agents such as curcumin [37] and auraptene [38] suppress iNOS activity. Our present study showed that nitrative guanosine modification may be mutagenic in mammalian cells. Therefore, further clarification of molecular mechanisms regulating formation and repair of 8-nitroguanosine is necessary for development of chemopreventive strategies against inflammation-associated carcinogenesis.

In conclusion, our data presented here provide experimental evidence that 8-nitroguanosine can serve as a mutagen in mammalian cells. Our study also showed that 8-nitroguanosine treatment significantly increased formation of abasic sites in cells.

These data, plus those previously reported on significant immunostaining for 8-nitroguanine in various cancer-related inflammatory diseases, indicate that 8-nitroguanosine may play an important role in inflammation-associated mutagenesis and carcinogenesis. Additional study is required to clarify whether tissue levels of 8-nitroguanosine are correlated with the degree of abasic site formation in cancer-associated inflammatory conditions.

Acknowledgments

We thank Ms. Judith B. Gandy for excellent editing the manuscript. We are also grateful to Dr. Marshall V. Williams, The Ohio State University, Columbus, OH, for providing the AS52 cells and to Mr. Takahiro Seki for technical support. This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and the Ministry of Health Labor and Welfare of Japan.

References

[1] F. Balkwill, A. Mantovani, Inflammation and cancer: back to Virchow?, Lancet 357 (2001) 539–545.
[2] L.M. Coussens, Z. Werb, Inflammation and cancer, Nature 420 (2002) 860–867.
[3] D. Schottenfeld, J. Beebe-Dimmer, Chronic inflammation: a common and important factor in the pathogenesis of neoplasia, CA Cancer J. Clin. 56 (2006) 69–83.
[4] H. Ohshima, M. Tatemichi, T. Sawa, Chemical basis of inflammation-induced carcinogenesis, Arch. Biochem. Biophys. 417 (2003) 3–11.
[5] T. Akuta, M.H. Zaki, J. Yoshitake, T. Okamoto, T. Akaike, Nitrative stress through formation of 8-nitroguanosine: insights into microbial pathogenesis, Nitric Oxide 14 (2006) 101–108.
[6] T. Sawa, H. Ohshima, Nitrative DNA damage in inflammation and its possible role in carcinogenesis, Nitric Oxide 14 (2006) 91–100.
[7] P.C. Dedon, S.R. Tannenbaum, Reactive nitrogen species in the chemical biology of inflammation, Arch. Biochem. Biophys. 423 (2004) 12–22.
[8] H. Ohshima, T. Sawa, T. Akaike, 8-Nitroguanine, a product of nitrative DNA damage caused by reactive nitrogen species: formation, occurrence, and implications in inflammation and carcinogenesis, Antioxid. Redox Signal. 8 (2006) 1033–1045.
[9] T. Akaike, S. Okamoto, T. Sawa, J. Yoshitake, F. Tamura, K. Ichimori, K. Miyazaki, K. Sasamoto, H. Maeda, 8-Nitroguanosine formation in viral pneumonia and its implication for pathogenesis, Proc. Natl. Acad. Sci. USA 100 (2003) 685–690.
[10] Y. Terasaki, T. Akuta, M. Terasaki, T. Sawa, T. Mori, T. Okamoto, M. Ozaki, M. Takeya, T. Akaike, Guanine nitration in idiopathic pulmonary fibrosis and its implication for carcinogenesis, Am. J. Respir. Crit. Care Med. 174 (2006) 665–673.
[11] T. Sawa, M. Tatemichi, T. Akaike, A. Barbin, H. Ohshima, Analysis of urinary 8-nitroguanine, a marker of nitrative nucleic acid damage, by high-performance liquid chromatography-electrochemical detection coupled with immunoaffinity purification: association with cigarette smoking, Free Radic. Biol. Med. 40 (2006) 711–720.
[12] N. Ma, Y. Adachi, Y. Hiraku, N. Horiki, S. Horiike, I. Imoto, S. Pinlaor, M. Murata, R. Semba, S. Kawanishi, Accumulation of 8-nitroguanine in human gastric epithelium induced by Helicobacter pylori infection, Biochem. Biophys. Res. Commun. 319 (2004) 506–510.
[13] S. Pinlaor, B. Sripa, N. Ma, Y. Hiraku, P. Yongvanit, S. Wongkham, C. Pairojkul, V. Bhudhisawasdi, S. Oikawa, M. Murata, R. Semba, S. Kawanishi, Nitrative and oxidative DNA damage in intrahepatic cholangiocarcinoma patients in relation to tumor invasion, World J. Gastroenterol. 11 (2005) 4644–4649.
[14] S. Horiike, S. Kawanishi, M. Kaito, N. Ma, H. Tanaka, N. Fujita, M. Iwasa, Y. Kobayashi, Y. Hiraku, S. Oikawa, M. Murata, J. Wang, R. Semba, S. Watanabe, Y. Adachi, Accumulation of 8-nitroguanine in the liver of patients with chronic hepatitis C, J. Hepatol. 43 (2005) 403–410.
[15] V. Yermilov, J. Rubio, H. Ohshima, Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination, FEBS Lett. 376 (1995) 207–210.
[16] N. Suzuki, M. Yasui, N.E. Geacintov, V. Shafirovich, S. Shibutani, Miscoding events during DNA synthesis past the nitration-damaged base 8-nitroguanine, Biochemistry 44 (2005) 9238–9245.
[17] M. Masuda, H. Nishino, H. Ohshima, Formation of 8-nitroguanosine in cellular RNA as a biomarker of exposure to reactive nitrogen species, Chem. Biol. Interact. 139 (2002) 187–197.
[18] J. Yoshitake, T. Akaike, T. Akuta, F. Tamura, T. Ogura, H. Esumi, H. Maeda, Nitric oxide as an endogenous mutagen for Sendai virus without antiviral activity, J. Virol. 78 (2004) 8709–8719.
[19] T. Sawa, T. Akaike, K. Ichimori, T. Akuta, K. Kaneko, H. Nakayama, D.J. Stuehr, H. Maeda, Superoxide generation mediated by 8-nitroguanosine, a highly redox-active nucleic acid derivative, Biochem. Biophys. Res. Commun. 311 (2003) 300–306.
[20] K.R. Tindall, L.F. Stankowski Jr., R. Machanoff, A.W. Hsie, Detection of deletion mutations in pSV2gpt-transformed cells, Mol. Cell. Biol. 4 (1984) 1411–1415.
[21] M.E. Ariza, M.V. Williams, Lead and mercury mutagenesis: type of mutation dependent upon metal concentration, J. Biochem. Mol. Toxicol. 13 (1999) 107–112.
[22] K.K. Richardson, J. Fostel, T.R. Skopek, Nucleotide sequence of the xanthine guanine phosphoribosyl transferase gene of *E. coli*, Nucleic Acids Res. 11 (1983) 8809–8816.
[23] H. Atamna, I. Cheung, B.N. Ames, A method for detecting abasic sites in living cells: age-dependent changes in base excision repair, Proc. Natl. Acad. Sci. USA 97 (2000) 686–691.
[24] H. Kasai, Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative