Niles *et al.* (48) studied the reaction of 2′,3′,5′-tri-*O*-acetylguanosine with peroxynitrite and identified 2′,3′,5′-tri-*O*-acetyl-8-nitroguanosine and 5-guanidino-4-nitroimidazole nucleoside, in addition to some oxidized guanine derivatives (Fig. 1). Some recent reviews (13, 67) have described in more detail other products of peroxynitrite-induced oxidative DNA damage, including 8-oxo-7,8-dihydroguanine (8-oxo-G), spiroiminodihydantoin, and other ring-cleavage products such as cyanuric acid and oxaluric acid nucleosides.

Formation of 8-NO_2-G by the reaction of guanine and its nucleosides with other RNS

Byun *et al.* (8) carried out the reaction of dGuo (2 m*M*) with a myeloperoxidase (MPO, 20 n*M*)–H_2O_2 (100 μ*M*)–nitrite (30 μ*M*) system at pH 7.4 and 37°C for 30 min and identified the two major reaction products as 8-NO_2-G and 8-NO_2-dGuo (total yield, 1.3 μ*M*). 8-NO_2-G could be formed from 8-NO_2-dGuo by spontaneous hydrolysis. Formation of nox-dGuo was not detected. With the MPO–H_2O_2–nitrite system, the presence of chloride ion did not affect the MPO-catalyzed nitration of dGuo, suggesting that hypochlorous acid (HOCl), a major product of the MPO–H_2O_2–Cl^- system, does not contribute to the nitrite-dependent nitration of dGuo by MPO. Human neutrophils stimulated with β-phorbol myristate acetate (PMA) also generated 8-NO_2-G and 8-NO_2-dGuo. The reaction required nitrite and was inhibited by catalase and heme poisons, indicating that MPO generated RNS that nitrate the C-8 position of dGuo in the cell-mediated pathway. Similarly, Masuda *et al.* (42) reported that Guo reacted with human MPO in the presence of nitrite, H_2O_2 and Cl^- or PMA-activated human neutrophils in the presence of nitrite and Cl^- to form 8-NO_2-Guo, in addition to 8-chloroguanosine and 8-oxo-7,8-dihydroguanosine (8-oxo-Guo).

Chen *et al.* (11) reported that nitryl chloride (NO_2Cl), prepared by mixing solutions of nitrite and HOCl, can react with guanine and xanthine to form the nitrated derivatives 8-NO_2-G and 8-nitroxanthine (8-NO_2-X), respectively. The reaction of nitryl chloride with dGuo generated both 8-NO_2-G and 8-NO_2-X. The same authors also showed formation of 8-NO_2-X by reactions of xanthine with large excess concentrations of other nitrating agents such as nitronium tetrafluoroborate and heated nitric and nitrous acids. Lin *et al.* (37) showed that peroxyacetyl nitrate [$CH_3C(=O)OONO_2$], a common gaseous photochemically generated compound in polluted air and cigarette smoke, nitrated guanine to form 8-NO_2-G. Yamada *et al.* (75) also showed formation of 8-NO_2-G *in vitro* by the reaction of dGuo with a gaseous mixture of NO and oxygen at pH 7.4 and 37°C, in addition to 8-NO_2-X, xanthine, deoxyxanthosine, and N^2-nitro-2′-deoxyguanosine. 8-NO_2-X has been reported to be formed by reactions of xanthine with peroxynitrite, the MPO–H_2O_2–nitrite system and a nitrating agent, tetranitromethane (63, 77). Thus, various nitrating agents can nitrate guanine and xanthine to form 8-NO_2-G and 8-NO_2-X *in vitro*.

The following mechanism has been proposed for the formation of 8-NO_2-G and 5-guanidino-4-nitroimidazole in the reaction of guanine with RNS (43, 48) (Fig. 1): (a) one-electron oxidation of guanine by oxidants yields a guanine radical [G(−H)•], which has significant unpaired electron density at the O6, C5, and C8 positions, and (b) radical combination between $•NO_2$ and the C8 or C5 positions of G(−H)• produces 8-NO_2-G or 5-nitro-guanine, respectively. The latter compound is then hydrolyzed, followed by C5–C6 bond cleavage, yielding 5-guanidino-4-nitroimidazole.

SYNTHESIS AND ANALYSIS OF 8-NO_2-G AND ITS DERIVATIVES

Synthesis

Several methods have been used to synthesize authentic 8-NO_2-G and 8-NO_2-Guo. The simplest is the reaction of guanine or Guo with peroxynitrite (78, 83). Bicarbonate, which catalyzes nitration reactions, can be added to increase the yield (80). 8-NO_2-G has been also prepared by the reaction of 8-bromoguanine with sodium nitrite in DMSO at 160°C (70). Similarly, 8-NO_2-Guo was prepared from 8-bromoguanosine with sodium nitrite in DMSO (3) or in DMF (34). 8-NO_2-G can be also prepared by diazotization of 8-amino-G with sodium nitrite in the presence of acid (31, 63). In all cases, purification of the product with HPLC is required. UV absorption maxima for 8-NO_2-G (pH 5.5) at 210, 231, 258, and 393 nm and ϵ_{400} = 9,144 $M^{-1}cm^{-1}$ (pH 7.0) have been reported (11). A different value [ϵ_{398} = 4,100 $M^{-1}cm^{-1}$ (pH 7.0)] has also been reported (70).

Stability of 8-NO_2-G, 8-NO_2-Guo and 8-NO_2-dGuo

8-NO_2-G is relatively stable in the absence of oxidizing agents, compared to 8-NO_2-Guo and 8-NO_2-dGuo. Aqueous solutions of 8-NO_2-G can be stored at 4°C for several months. However, Burney *et al.* (7) reported that 8-NO_2-G can be more easily further oxidized with peroxynitrite than 8-oxo-dGuo or dGuo, yielding some oxidized products including 8-oxo-guanine (8-oxo-G) (34). On the other hand, 8-NO_2-dGuo and 8-NO_2-Guo are less stable than 8-NO_2-G. Sodum and Fiala (63) reported the half-life of 8-NO_2-dGuo to be 44 h at −20°C, 7 h at 0°C, about 10 min at room temperature, and <3 min at 37°C. In contrast, 8-NO_2-Guo is more stable, with a half-life of several weeks at 5°C and about 5 h at 37°C.

Analysis of 8-NO_2-G and 8-NO_2-Guo

8-NO_2-G and 8-NO_2-Guo are yellow, having UV absorption around 390 nm, and thus can be measured with a UV/visible spectrophotometer or a photodiode array detector. Early studies (11, 64) used such methods. However, the methods appear not to be sensitive enough to analyze these adducts in biological specimens.

8-NO_2-G, 8-NO_2-X, and 8-NO_2-Guo can be measured by HPLC with electrochemical detection. Although high oxidative potentials (>0.8 V) are required to detect these nitrated adducts by electrochemical detectors, their reduction products, [i.e., 8-amino-G, 8-aminoxanthine and 8-aminoguanosine (8-amino-Guo)] can be detected at oxidative potentials as low as 0.2 V. The use of such low potentials has the advantage of providing increased selectivity and lower detection limits during the analysis of biological samples, since fewer interfering compounds will be oxidized under these conditions. For this purpose, nitrated derivatives are chemically re-

duced to their amino derivatives with sodium hydrosulfite (78, 79) or reduced on-line directly with electrodes in reductive mode (53, 63). The methods have been successfully used to detect 8-NO_2-G and 8-NO_2-X in biological specimens such as urine (see below).

MS methods such as electrospray MS have been used to confirm the structures of reaction products of guanine and its nucleosides with RNS (11, 48, 78). A gas chromatography–MS method was also used to study products formed from dGuo with the MPO–H_2O_2–nitrite system (8). The products, including 8-NO_2-G and 8-amino-G, were converted to their trimethylsilyl or tert-butyldimethylsilyl derivatives before gas chromatography. An HPLC–negative ion electrospray ionization MS method with selected reaction monitoring mode has been also used to analyze 8-NO_2-G with ^{15}N,^{13}C-8-NO_2-G as an internal standard (70). In view of rapid developments in the area of MS analytical instruments, such methods are a promising approach for selective and sensitive analysis of 8-NO_2-G in biological specimens.

When 8-NO_2-G in DNA or RNA is analyzed (see below), DNA or RNA samples should be hydrolyzed into bases or nucleosides. 8-NO_2-G in DNA is hydrolyzed by heating in acids [0.1 *M* HCl at 100°C for 60 min (79), 60% (v/v) formic acid at 150°C for 45 min (64) or formic acid containing butylated hydroxytoluene at 130°C for 30 min (71)] or under neutral conditions (5 min at 90°C) (70). As 8-NO_2-Guo is stable, the RNA samples can be hydrolyzed enzymatically to nucleosides using nuclease P1 and acid phosphatase at 37°C for 30 min (41).

FORMATION OF 8-NO_2-G IN DNA AND RNA *IN VITRO*

Reactions of isolated DNA and RNA with various RNS

Yermilov *et al.* (79) first reported in 1995 that 8-NO_2-G is formed dose-dependently in calf-thymus DNA incubated with low concentrations of peroxynitrite *in vitro*. When calf-thymus DNA (0.2 mg/ml) was incubated with 5–100 μ*M* peroxynitrite at pH 7 and room temperature, about 400–2100 residues of 8-NO_2-G per 10^6 guanine were formed. Other groups reported similar findings on 8-NO_2-G formation in peroxynitrite-treated DNA, although the amounts varied considerably. Spencer *et al.* (64) reported that very high concentrations of 8-NO_2-G (about 120 nmol/mg of DNA, corresponding to about 30,000 adducts per 10^6 DNA bases) were formed in calf-thymus DNA (0.2 mg/ml) incubated with 1 m*M* peroxynitrite at pH 7. Tuo *et al.* (71) reported about 100–400 8-NO_2-G per 10^6 guanine in calf-thymus DNA (0.2 mg/ml) incubated with 100–500 μ*M* peroxynitrite and also found that large amounts of 8-NO_2-G were released into the medium during the reaction. Reasons for the varying yields of 8-NO_2-G in peroxynitrite-treated DNA are not clear. The articles by Yermilov *et al.* (79), Spencer *et al.* (64), and Tuo *et al.* (71) reported that levels of an oxidized DNA base, 8-oxo-G, in their peroxynitrite-treated DNA samples also varied considerably. The ratios of 8-NO_2-G to 8-oxo-G were 5 (79), 100 (64), and 0.1 (71). These variations may be attributed to (a) different peroxynitrite preparations (contamination with hydrogen peroxide, nitrite, metal ions, and others), (b) experimental conditions, especially reaction pH and dose and dose rate of peroxynitrite, and (c) experimental procedures preceding the analysis of 8-NO_2-G (precipitation and hydrolysis of DNA after the reaction). Sodum and Fiala (63) reported that more 8-oxo-dGuo than 8-NO_2-G plus 8-NO_2-dGuo was formed under acidic pH (< 5), but more 8-NO_2-G plus 8-NO_2-dGuo than 8-oxo-dGuo under neutral pH in the reaction of dGuo with peroxynitrite. It should also be noted that 8-NO_2-G in DNA is not stable and can be lost during DNA precipitation (see below).

In addition to peroxynitrite, other RNS-generating systems can nitrate guanine residues in DNA. Tuo *et al.* (71) reported that 8-NO_2-G levels increased dose-dependently with 30–90 n*M* MPO in calf-thymus DNA (0.2 mg/ml) incubated in the presence of 150 μ*M* H_2O_2 and 60 n*M* nitrite (pH 7.4) at 37°C for 30 min (7–19 adducts per 10^6 guanine). Human neutrophils activated with PMA also nitrated calf-thymus DNA (0.2 mg/ml) to yield 9 residues of 8-NO_2-G per 10^6 guanine in the presence of 60 n*M* nitrite at 37°C for 60 min. Chen *et al.* (11) reported that both 8-NO_2-G and 8-NO_2-X are formed in DNA incubated with a large excess (250 times) of nitryl chloride, the yields being 0.71% and 0.30% of guanine residues in DNA or 7,100 and 3,000 adducts per 10^6 guanine, respectively. In this paper, however, it was not clearly described whether artifactual formation of 8-NO_2-X during DNA hydrolysis was prevented: nitrite easily deaminates 8-NO_2-G to 8-NO_2-X during acid hydrolysis at high temperature. Hsieh *et al.* (27) reported dose-dependent formation of 8-NO_2-G (2.5–5 nmol per mg DNA, corresponding to about 600–1200 adducts per 10^6 bases) in DNA (0.2 mg/ml, isolated from human lung fibroblasts, MRC-5) incubated with 2–20 μ*M* gaseous NO at 37°C for 48 h.

Gu *et al.* (20) reported that the reaction of calf-thymus DNA incubated with low concentrations of peroxynitrite formed 5-guanidino-4-nitroimidazole dose-dependently, along with several other products. Joffe *et al.* (30) showed that the ratio of the 5-guanidino-4-nitroimidazole to 8-NO_2-G lesions was about 1 in double-stranded oligodeoxynucleotides nitrated photochemically in the presence of bicarbonate and nitrite.

Masuda *et al.* (41) studied 8-NO_2-G formation in RNA with various RNS. 8-NO_2-G in RNA was found to be much more stable than 8-NO_2G in DNA (see below). When calf-liver RNA (1 mg/ml) was incubated with 0.5 m*M* peroxynitrite, about 110 8-NO_2-Guo per 10^6 Guo were formed. Similarly, when RNA (1 mg/ml) was incubated at 37°C for 1 h with 0.5 m*M* 3-morpholinosydnonimine (SIN-1), which spontaneously decomposes to release both NO and superoxide, thus forming peroxynitrite (21, 25), 15 8-NO_2G per 10^6 Guo were generated. Nitrite plus HOCl (both at 0.5 m*M*), which react together to form nitryl chloride (18, 54), formed small amounts (12 adducts per 10^6 Guo) and human MPO and horseradish peroxidase catalyzed the formation of 9 and 7 adducts per 10^6 Guo, respectively, in the presence of nitrite and H_2O_2.

Modification of 8-NO_2-G formation by CO_2 and antioxidants

Nitration and oxidation mediated by peroxynitrite and other RNS are modified by a variety of compounds. Peroxy-

nitrite reacts with carbon dioxide (CO_2) with a rate constant of $5.8 \times 10^4\ M^{-1}\ s^{-1}$ to form the intermediate nitrosodioxycarboxylate ($O = NOOCOO^-$), which can decompose to reactive intermediates such as $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ radicals or rearrange to nitrooxo-carboxylate anion ($O_2N\text{-}O\text{-}CO_2^-$) (14, 38). Thus, the presence of CO_2, added as 0–10 m*M* bicarbonate, caused a dose-dependent increase of up to sixfold in the peroxynitrite-mediated formation of 8-NO_2-G in DNA and RNA (41, 80). As the concentrations of CO_2 (1.3 m*M* in blood plasma) and bicarbonate (12 m*M* in intracellular fluid and 25–30 m*M* in blood plasma) are high, this reaction between peroxynitrite and CO_2 occurs *in vivo* and enhances nitration reactions (73). On the other hand, Fe^{3+}-EDTA, which enhances nitration of tyrosine and phenolics by peroxynitrite (4), as well as hydroxyl radical scavengers such as DMSO, ethanol, and D-mannitol did not affect the formation of 8-NO_2-G in DNA (79). Conversely various antioxidants, such as *N*-acetylcysteine, uric acid, and ascorbic acid, an iron chelator (desferrioxamine), and several flavonoids and other phenolic compounds can prevent formation of 8-NO_2-G in DNA by RNS such as peroxynitrite or nitryl chloride (12, 53, 79, 83).

Formation of Guo adducts (8-NO_2-Guo, 8-oxo-Guo and 8-chloroguanosine) with the MPO–H_2O_2–nitrite–Cl^- system was dramatically enhanced by low concentrations of tertiary amines such as nicotine and trimethylamine (42). On the other hand, taurine and methionine, two potent scavengers of HOCl, failed to inhibit nitration of 2′-deoxyguanosine by this system (8).

Stability of 8-NO_2-G in nitrated DNA and RNA

Yermilov *et al.* (79) observed that upon incubation of DNA containing 8-NO_2-G (prepared by the reaction with peroxynitrite) at 37°C and pH 7.4, 8-NO_2-G disappeared rapidly from DNA and free 8-NO_2-G appeared in the medium. They estimated the half-life of 8-NO_2-G in DNA to be about 4 h. Chen *et al.* (9) observed even faster depurination of 8-NO_2-G from peroxynitrite-treated calf-thymus DNA at 37°C and pH 7.0–7.5, with a half-life of about 1 h (estimated from Fig. 7 in Ref. 9). This spontaneous depurination of 8-NO_2-G in DNA has been confirmed using oligodeoxynucleotides treated with peroxynitrite or synthetic oligodeoxynucleotides containing 8-NO_2-G at a specific site. Tretyakova *et al.* (70) estimated the rate of formation of spontaneously generated abasic sites in peroxynitrite-treated pUC19 plasmid to be about 1 h at 37°C (the abasic sites were presumably generated by release of 8-NO_2-G). Shafirovich *et al.* (61) synthesized an oligodeoxynucleotide containing 8-NO_2-G at a specific site and measured their rates of spontaneous depurination. The nitrated oligodeoxynucleotide released free 8-NO_2-G and formed an abasic site at this position, with a half-life of about 20 h at 23°C and pH 7. Suzuki *et al.* (66) estimated the half-life of another oligodeoxynucleotide containing 8-NO_2-G at a specific site to be 31 h at 25°C.

Chen *et al.* (11) reported depurination rates of 8-NO_2-X from calf thymus DNA treated with nitryl chloride to be about 2 h. On the other hand, 5-guanidino-4-nitroimidazole in DNA is much more stable than 8-NO_2-G and 8-NO_2-X [10–15% decomposition at 90°C for 6 h and only partial decomposition after hot piperidine treatment (1 *M* piperidine for 30 min at 90°C)] (20, 30).

While 8-NO_2-G in DNA is spontaneously depurinated with a half-life of 1–4 h at 37°C and pH 7.4, Masuda *et al.* (41) found that 8-NO_2-G present in RNA is relatively stable, with only about 5% of the modified nucleoside lost during 6 h of incubation under similar conditions.

Repair of 8-NO_2-G lesions in DNA

It is not known whether 8-NO_2-G in DNA can be enzymatically repaired. Two studies have shown that 8-NO_2-G in DNA was not recognized by formamidopyrimidine glycosylase (Fpg), a DNA repair enzyme with glycosylase and abasic (AP) endonuclease activity against a broad range of oxidized purines, including 8-oxo-G and 2,6-diamino-5-formamidopyrimidine. Tretyakova *et al.* (70) reported that 8-NO_2-G in a peroxynitrite-treated oligodeoxynucleotide (13-mer) was not recognized by Fpg. In agreement with this observation, Tuo *et al.* (71) reported that incubation of peroxynitrite- or MPO–H_2O_2–nitrite-treated calf-thymus DNA with Fpg released 8-oxo-G, but not 8-NO_2-G. These results suggest that 8-NO_2-G is not a substrate for Fpg.

Enzymatic reduction of 8-NO_2-G

Chen *et al.* (10) reported that 8-NO_2-G and 8-NO_2-X in free form or in DNA can be reduced to their amino derivatives by lipoyl dehydrogenase (EC 1.8.1.4) from *Clostridium kluyveri* and from porcine heart using NAD(P)H as a cofactor. Similarly, Chen *et al.* (9) found that hemin and hemoproteins, including hemoglobin and cytochrome *c*, mediate the same reduction of 8-NO_2-G and 8-NO_2-X in free form or in DNA in the presence of reducing agents such as ascorbate and glutathione. The biological significance of these findings is unknown, but the authors suggested that these enzymatic reductions might represent a metabolic pathway to reverse the process of biological nitration.

FORMATION OF 8-NO_2-G IN DNA AND RNA IN CELLS AND TISSUES

Formation in cultured cells exposed to RNS

We attempted to analyze 8-NO_2-G in DNA isolated from tissues and cells. However, due to its instability in DNA (see above), we found that 8-NO_2-G was lost during extraction of DNA, so that its measurement in cellular DNA as an exposure marker was not possible (Yermilov *et al.*, unpublished data). Nevertheless several articles have reported the occurrence and formation of 8-NO_2-G in DNA extracted from cultured cells, animal tissues, and human blood lymphocytes, but it should be noted that these results have not been reproduced and/or confirmed by other groups.

Spencer *et al.* (64) incubated human keratinocytes in Hank's balanced salt solution with 1 m*M* peroxynitrite with constant mixing for 10 s (final pH 7.2) at 37°C. DNA was extracted and hydrolyzed in 60% formic acid for 45 min at 150°C. Analysis by HPLC with a UV detector at 396 nm showed about 8 nmol 8-NO_2-G/mg DNA (corresponding to

about 2,000 adducts per 10^6 DNA bases). Similarly, incubation of cells at 37°C for 60 min with 1 m*M* SIN-1, a peroxynitrite generator, yielded about 10 nmol 8-NO_2-G/mg DNA (corresponding to 2,500 8-NO_2-G per 10^6 DNA bases).

Hsieh *et al.* (27) reported a time- and dose-dependent increase in 8-NO_2-G formation in DNA of human lung fibroblasts (MRC-5) exposed to NO. The cells were exposed to NO gas-saturated PBS with initial NO concentrations of 2–20 μ*M* for 0–48 h. DNA was extracted from the cells and acid-hydrolyzed. 8-NO_2-G was determined by HPLC with a UV detector at 254 nm. The levels of 8-NO_2-G ranged from 2.4 to 3.8 nmol/mg DNA (corresponding to ~600 to ~900 adducts per 10^6 DNA bases).

Although the above studies reported formation of 8-NO_2-G in DNA following exposure of cells to RNS in culture, we have not been able to reproduce these results in our laboratory, mainly due to the instability of 8-NO_2-G during extraction of DNA from cells, as described above. On the other hand, Masuda *et al.* (41) detected 7–50 8-NO_2-Guo per 10^6 Guo in RNA isolated from human lung carcinoma (A549) cells (4×10^6/20 ml PBS) incubated with synthetic peroxynitrite (1 m*M*, final pH 7.3) at 37°C for 5 min. 8-NO_2-Guo in enzymatic hydrolyzates of RNA was converted with sodium hydrosulfite to 8-amino-Guo, which was then analyzed by HPLC with an electrochemical detector.

Detection of 8-NO_2-G in DNA isolated from animal and human tissues

Tuo *et al.* (71) first attempted to analyze 8-NO_2-G in liver DNA of mice treated intraperitoneally with *Escherichia coli* lipopolysaccharide (LPS) (5 mg per kg b.w.). The liver was removed 6 h after LPS administration and DNA was extracted. 8-NO_2-G in acid-hydrolyzed DNA was converted chemically (sodium hydrosulfite) to 8-amino-G, followed by detection by HPLC with an electrochemical detector. The authors reported finding 8-NO_2-G in the liver DNA from 2 out of 12 treated mice at levels of 5 and 4 adducts per 10^6 guanine, levels close to the detection limit. However, under this experimental protocol, induction of an inducible type of nitric oxide synthase (iNOS), measured as serum nitrate/nitrite, was marginal at 6 h after LPS treatment (72). The stability and recovery of 8-NO_2-G in DNA during isolation from the liver were not examined.

Hsieh *et al.* (26) reported the levels of 8-NO_2-G in DNA extracted from peripheral lymphocytes of 15 each of nonsmokers, slight, moderate, and heavy smokers and lung cancer patients with heavy smoking. 8-NO_2-G in acid-hydrolyzed DNA was analyzed by HPLC with an electrochemical detector. DNA from nonsmokers contained only trace levels of 8-NO_2-G (0.02 μg/mg DNA or 25 adducts per 10^6 DNA bases), but levels were significantly higher in DNA from heavy smokers and lung cancer patients with heavy smoking (about 1.2 and 1.4 μg/mg DNA; ~1500 and ~1800 adducts per 10^6 DNA bases, respectively). The same authors also reported a dose-dependent increase in 8-NO_2-G in DNA of the lung and peripheral lymphocytes of Wistar rats exposed to cigarette smoke twice a day for one month. However, the experimental procedures (the volume of blood taken for extraction of DNA, conditions for electrochemical detection of 8-NO_2-G. etc.) were not described in detail, and the stability and recovery of 8-NO_2-G in DNA during isolation from the lung and peripheral lymphocyte were not examined. Further studies are needed to confirm these preliminary findings.

Occurrence of 8-NO_2-G and 8-NO_2-X in human urine

Sawa *et al.* (60) recently developed an analytical method to measure 8-NO_2-G and 8-NO_2-X in human biological specimens such as urine. Human urine (50 ml), to which 500 dpm [^{14}C]-8-NO_2-G had been added as an internal standard, was purified by an immunoaffinity column prepared with a monoclonal antibody against 8-NO_2-G (see below). 8-NO_2-G and 8-NO_2-X in the purified urine extract were analyzed by HPLC with an ESA Coul-Array electrochemical detector. Four electrodes were sequentially used at +250, −1,000, −1,000, and +150 mV. Some interfering compounds, if present in the sample, could be oxidized at the first electrode. Nitrated bases were then reduced on-line with two electrodes under a reduction mode at −1,000 mV and the reduced derivatives were analyzed quantitatively with the fourth electrode at +150 mV. This method is sensitive and specific for the analysis of 8-NO_2-G and 8-NO_2-X, with detection limits for both compounds of 25–50 fmol/injection. We have used it to detect significantly increased levels of 8-NO_2-G and 8-NO_2-X in urine from smokers [median of 8-NO_2-G plus 8-NO_2-X (range): 10.0 (0–29.5) fmol/mg creatinine, $n = 12$] compared to those from nonsmokers [0 (0–5.4) fmol/mg creatinine, $n = 17$]. This is the first finding of 8-NO_2-G and 8-NO_2-X in human urine.

IMMUNOHISTOCHEMICAL DETECTION OF 8-NO_2-G

Production of polyclonal and monoclonal antibodies against 8-NO_2-Guo and their application to study 8-NO_2-Guo formation in cultured cells and in RNA virus-induced pneumonia in mice

Akaike *et al.* (3) were the first to prepare polyclonal antibodies against 8-NO_2-Guo in rabbits using 8-NO_2-Guo-bovine serum albumin (BSA) conjugate as antigen. The antibody was purified by use of a series of affinity chromatographic procedures. Competitive enzyme immunoassay showed that only 8-NO_2-G and 8-NO_2-Guo, not guanine, Guo, 8-oxo-G, 8-oxoGuo, or 3-nitrotyrosine, completely inhibited binding between the purified antibody and the 8-NO_2-Guo–BSA conjugate. In slot blot analyses, an RNA sample treated with peroxynitrite *in vitro* showed strong immunoreactivity with the antibody. The immunoreactivity was also detected against total RNA extracted from RAW 264 cells that had been stimulated with LPS and interferon-γ to induce iNOS, and was eliminated almost totally when the RNA sample was treated with sodium hydrosulfite, which converts 8-NO_2-Guo to 8-amino-Guo. On the other hand, total RNA samples isolated from nonstimulated cells or stimulated cells

cultured in the presence of an NO synthase inhibitor, N^{ω}-monomethyl-L-arginine, showed lower immunoreactivity. These results indicated that the antibody could recognize 8-NO_2-Guo in RNA and that an appreciable amount of 8-NO_2-Guo was endogenously formed in cells producing NO.

Akaike *et al.* (3) applied this antibody against 8-NO_2-Guo to study formation of 8-NO_2-Guo *in vivo* in mice infected with influenza or Sendai viruses. Infection with these viruses induces pneumonia in mice and its lethal effects could be mediated by reactive oxygen species and RNS produced as a host response (1, 49). Strong 8-NO_2-Guo immunostaining was observed primarily in the cytosol of bronchial and bronchiolar epithelial cells of virus-infected wild-type mice 6–8 days after infection. This staining colocalized with iNOS immunostaining, particularly in bronchial cells, and correlated well with formation of 3-nitrotyrosine, a marker of protein nitration. The 8-NO_2-Guo immunostaining was prevented by preabsorption of the antibody with free 8-NO_2-Guo or pretreatment of tissues with sodium hydrosulfite. On the other hand, 8-NO_2-Guo immunostaining was absent in airways of iNOS-deficient mice, in which the lethal effects of viral infection were markedly weaker.

Akaike's group recently produced a mouse monoclonal antibody against 8-NO_2-Guo (81) using 8-NO_2-Guo–BSA conjugate as antigen. The specificity of this monoclonal antibody was confirmed by a competitive enzyme immunoassay, as described for production of the polyclonal antibody against 8-NO_2-Guo. The antibody could be used to immunolocalize 8-NO_2-Guo in the cytosol of bronchial and bronchiolar epithelial cells of virus infected mice (Fig. 2). Yoshitake *et al.* (81) also used this monoclonal antibody to immunolocalize 8-NO_2-Guo in human adenocarcinoma (SW480) cells with and without transfection of a rat iNOS expression vector. Much stronger 8-NO_2-Guo immunostaining was observed in iNOS-expressing SW480 cells than in cells without iNOS. Analysis by confocal laser scanning microscopy showed that 8-NO_2-Guo was localized mainly in the cytosol of iNOS-expressing SW480 cells. These findings are consistent with those from the study on formation of 8-NO_2-Guo *in vivo* in mice infected with influenza or Sendai viruses (3).

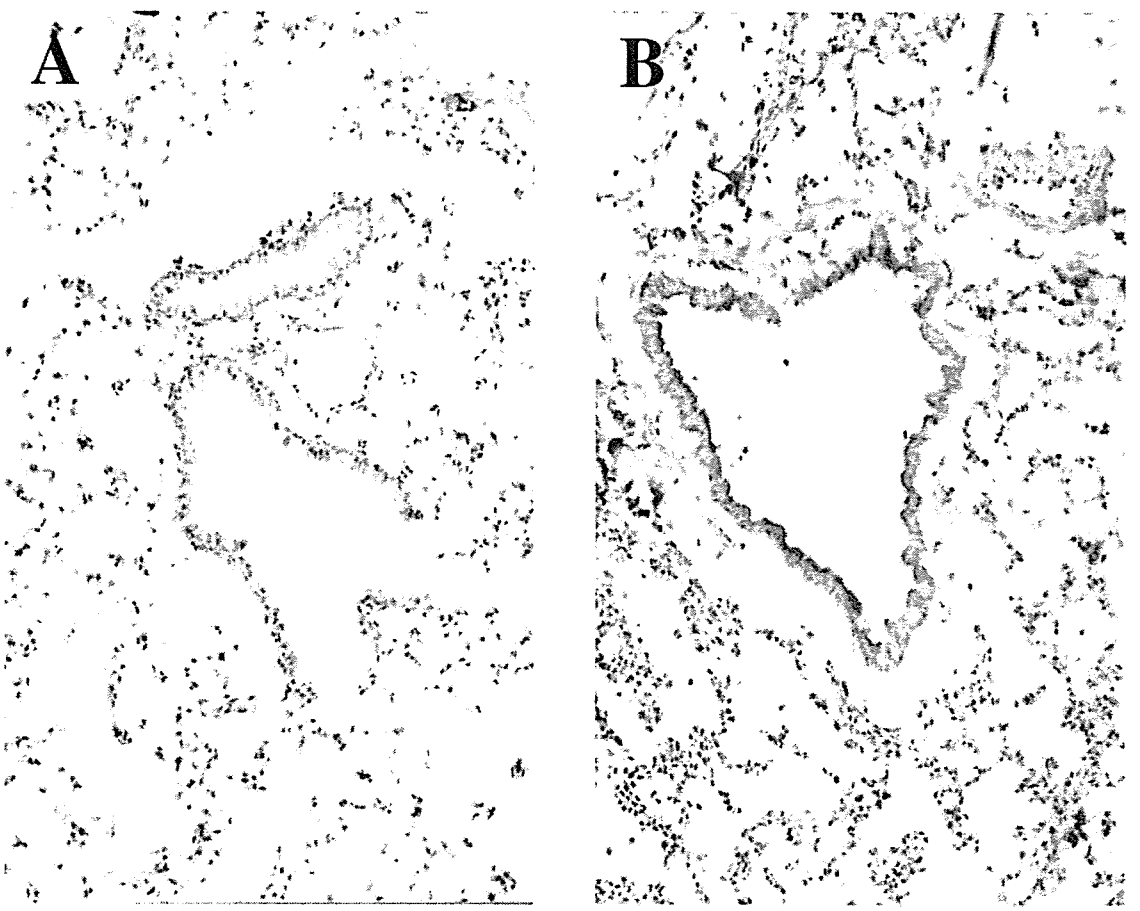

FIG. 2. Immunohistochemical detection of 8-NO_2-G *in vivo*. Mouse lung obtained at 0 (**A**) and 8 (**B**) days after influenza virus infection (3) was immunostained with anti-8-nitroguanosine monoclonal antibody (81).

The results from these studies in mice and iNOS-expressing cells indicate that NO production is needed for formation *in vivo* of 8-NO_2-Guo, which mainly localizes in the cytosol. This observation may be explained by the following: 8-NO_2-G in DNA is unstable and undergoes spontaneous depurination to produce an apurinic site (see above). Thus, 8-NO_2-G does not accumulate in DNA in an amount sufficient for detection by immunohistochemical analysis; 8-NO_2-Guo, however, is stably formed in the nucleotide pool and RNA in the cytosol of the cells (81).

The mouse monoclonal 8-NO_2-Guo antibody has also been used successfully to show that 8-NO_2-Guo is formed in mouse chondrocyte-like ATDC5 cells and rat primary chondrocytes after exposure to interleukin-1β, which acts as a key mediator of the degradation of articular cartilage in rheumatoid arthritis (76). Upon exposure to interleukin-1β, chondrocytes showed immunoreactivity against 8-NO_2-Guo, which was inhibited by N^{ω}-monomethyl-L-arginine, suggesting the production of RNS such as peroxynitrite.

Occurrence of 8-NO_2-G in inflamed tissues

Pinlaor *et al.* (57) applied a commercial anti-8-NO_2-G mouse monoclonal antibody [Dojindo Laboratories, Kumamoto, Japan; the same monoclonal antibody as reported by Yoshitake *et al.* (81)] to immunolocalize 8-NO_2-G in the liver of hamsters infected with the parasite *Opisthorchis viverrini* (OV). OV infection has been associated with increased risk of intrahepatic cholangiocarcinoma in northeast Thailand (28). Enhanced iNOS activity and NO production have been reported in the liver of hamsters infected with OV (50). 8-NO_2-G immunoreactivity was found mainly in the cytoplasm and slightly in the nucleus of inflammatory cells and epithelial lining of bile ducts in inflammatory areas of the liver of OV-infected hamsters. The immunoreactivity was highest 30 days after infection and then decreased until 60 days. It was absent in the liver of uninfected hamsters.

Kawanishi's group (55) recently produced a rabbit polyclonal antibody against 8-NO_2-G, using a similar approach to that of Akaike *et al.* (3). They performed double immunofluorescence staining of 8-NO_2-G and 8-oxo-dGuo in the liver of OV infected-hamsters, using this polyclonal antibody together with anti-8-oxo-dGuo mouse monoclonal antibody (69). Immunoreactivity for both 8-NO_2-G and 8-oxo-dGuo was observed mostly in the nucleus of the same inflammatory cells and in epithelium of bile ducts in livers of hamsters from day 7 after OV infection. Staining was maximal at 21–30 days, and lower at 90 and 180 days. Presence of 8-NO_2-G and 8-oxo-dGuo was also detected in some small bile ducts at day 180. The pattern of 8-NO_2-G staining in this study (mostly nuclear localization), however, appears to be different from that seen earlier by the same group using an 8-NO_2-G monoclonal antibody (mainly in the cytoplasm) (57).

Double immunofluorescence staining of 8-NO_2-G and 8-oxo-dGuo has been applied to examine several animal and human tissues, including the liver of hamsters infected repeatedly with OV (56), human gastric mucosa infected with *Helicobacter pylori* (39), and colon tissue from mice with inflammatory bowel diseases (15). In these studies, both 8-NO_2-G and 8-oxo-dGuo were detected mostly in the nucleus of inflammatory cells and/or epithelial cells in inflamed tissues, but not in normal tissues. Immunoreactivity was also spread in the cytoplasm and also weakly in mitochondria. Further studies using chromatographic methods are needed to confirm that 8-NO_2-G is indeed formed in the nucleus.

BIOLOGICAL SIGNIFICANCE

Superoxide generation mediated by 8-NO_2-Guo

Sawa *et al.* (58) and Akaike *et al.* (3) reported that 8-NO_2-Guo and its 5′-monophosphates and 5′-triphosphates are highly redox-active nucleic acid derivatives that strongly stimulate superoxide generation in the presence of various NADPH-dependent reductases, including cytochrome P450 reductase and all isoforms of NO synthase. These reductases in the presence of NADPH catalyze single-electron reduction of 8-NO_2-Guo to form 8-NO_2-Guo anion radical, from which one electron is then transferred to molecular oxygen to form superoxide in a redox cycling reaction. Thus, 8-NO_2-Guo and its related nucleotides may participate in diverse physiological and pathological events (Fig. 3).

Viral mutation induced by 8-NO_2-Guo

Yoshitake *et al.* (81) examined whether 8-NO_2-Guo can induce mutations in an RNA virus. They analyzed phenotypic alteration (loss of fluorescence) in Sendai virus fused to a green fluorescent protein gene (GFP–SeV) that propagated in cells or in mice (2). Authentic 8-NO_2-Guo added exogenously to GFP–SeV-infected CV-1 cells caused a dose-dependent increase in viral mutations, especially C to U transitions. The same type of mutation was also detected in GFP–SeV isolated from lungs of mice 7 days after infection with this virus. Similarly, the GFP–SeV mutation frequency was much higher in iNOS-expressing SW480 cells than in parent SW480 cells. As 8-NO_2-Guo was detected in the cytosol of iNOS-expressing SW480 cells, but not in parent cells (see above), the authors proposed that NO has mutagenic potential for RNA viruses such as Sendai virus, possibly via 8-NO_2-Guo formation and cellular oxidative stress due to production of superoxide mediated by 8-NO_2-Guo (see above).

Roles of 8-NO_2-G in gene mutation and carcinogenesis

As described above, 8-NO_2-G residues in DNA may be rapidly depurinated from DNA *in vitro* with a half-life of 1–4 h under physiological conditions, resulting in the formation of mutagenic abasic sites and release of free 8-NO_2-G. Thus, the formation of 8-NO_2-G in DNA may facilitate G to T transversion via abasic site formation (79). In addition, Suzuki *et al.* (66) recently synthesized an oligodeoxynucleotide containing a single 8-NO_2-dGuo at a specific position by photochemical synthesis (61) and used it as a template in primer extension reactions catalyzed by mammalian DNA polymerases. Primer extension reactions catalyzed by certain polymerases were strongly retarded at the 8-NO_2-dGuo lesion. However, a fraction of primers was extended past the lesion by incorporating adenine residues, in addition to the cor-

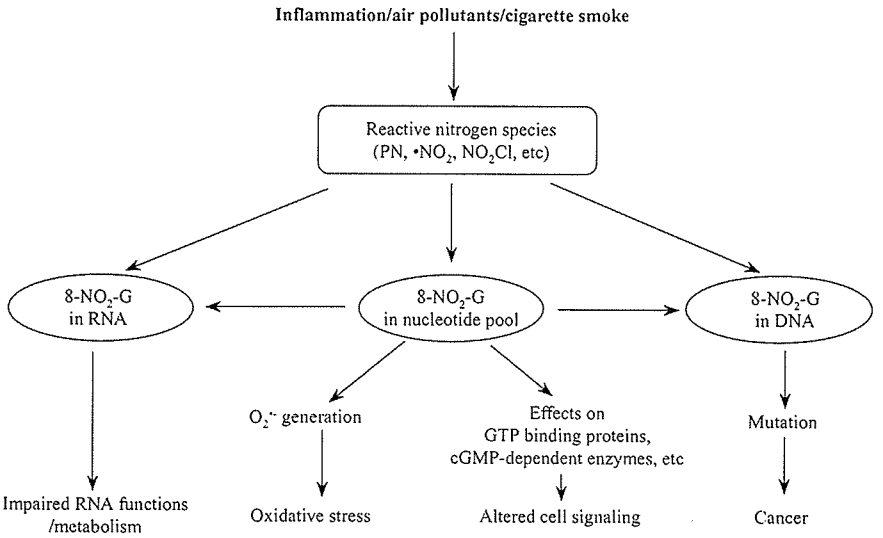

FIG. 3. Biological significance of 8-NO_2-G formed in a cell.

rect cytosine residues, opposite 8-NO_2-dGuo in an oligodeoxynucleotide. This suggests that 8-NO_2-dGuo in DNA can mispair with adenine, directly inducing a G to T transversion in mammalian cells. Neeley *et al.* (47) also studied the miscoding potential of 5-guanidino-4-nitroimidazole, another nitrated guanine derivative formed by the reaction with peroxynitrite, using M13mp7L2 bacteriophage genome containing the adduct at a known position, which was used to transform *E. coli* cells. Analyses of the resulting progeny phage showed that this nitrated adduct is a strong block to replication, and 50% mutagenic, generating G to A, G to T, and to a lesser extent, G to C mutations.

In agreement with these observations, peroxynitrite has been reported to be strongly mutagenic in the *supF* shuttle vector pSP 189 replicated in bacteria or human cells (32). The majority of mutations were found at G:C base pairs, predominantly involving G to T transversion, followed by G to C transversion and G to A transition.

As mentioned above, immunohistochemical studies have demonstrated that 8-NO_2-G is also present in the cytosol of NO-producing cells, suggesting that 8-NO_2-G may be produced in the nucleotide pool, RNA, and other compartments in the cytosol. Although certain enzymes such as MPO are unlikely to nitrate DNA directly, nitrated nucleic acids may be formed in the nucleotide pool or in RNA by RNS generated by such enzyme systems. As shown for some oxidized and halogenated nucleosides (23, 44, 45), 8-NO_2-G and related nucleosides and nucleotides may be misincorporated into DNA, which can result in mutations. Similarly, 8-NO_2-G and related nucleosides and nucleotides may be incorporated into RNA to interfere with RNA function and metabolism, as occurs with oxidized and halogenated nucleosides (36, 62, 68) (Fig. 3).

Chronic inflammation induced by a variety of biological, chemical, and physical factors has been associated with increased risk of human cancer at many sites (51, 52, 59). The studies summarized above clearly show that nitrative DNA damage can be induced under various inflammatory conditions, as evidenced by increased formation of 8-NO_2-G. However, further studies are required to establish a causal relationship between this type of DNA damage and human cancer, using a molecular epidemiological approach in a large human population.

Roles of 8-NO_2-G in cellular signaling

In addition to enzymatic superoxide generation mediated by various reductases in the presence of 8-NO_2-Guo and nitrated nucleotides, these nitrated derivatives may interfere with or modulate functions of various important enzymes that utilize GTP, GDP, GMP, and cGMP as substrates. Examples include various GTP-binding proteins including Ras proteins and various cGMP-dependent enzymes. Recently Heo *et al.* (24) reported that treatment of Ras with NO and oxygen caused conversion of Ras-bound GDP into a free 463.3 Da nucleotide-nitration product, which was identified as 5-guanidino-4-nitroimidazole diphosphate, a degradation product of 5-nitro-GDP. They proposed the mechanism that $^{\bullet}NO_2$ formed from NO and oxygen reacts with Ras to generate a Ras-Cys 118 thiyl radical intermediate, which withdraws an electron from the Ras-bound guanine nucleotide base to produce a guanine nucleotide diphosphate cation radical ($G^{\bullet+}$-DP), reacting with $^{\bullet}NO_2$ to form 5-nitro-GDP. This radical-based reaction process disrupts key binding interactions between Ras and the guanine base, resulting in release of GDP from Ras and its conversion to free 5-nitro-GDP. This mechanism may be common to other Ras superfamily GTPases. Further studies are warranted to investigate effects of nitrated nucleotides on these enzymes in relation to intracellular signaling systems.

CONCLUSION

We have reviewed studies on 8-NO_2-G published in the last decade. Various RNS produced in a variety of pathophysiological conditions may nitrate guanine and its related nucleosides and nucleotides in the free form, or in DNA or RNA. Immunohistochemical studies with 8-NO_2-G specific antibodies have shown that the adduct is formed in the cytosol as well as in the nucleus of inflammatory and epithelial cells in inflamed tissues, but not in normal tissues. 8-NO_2-G in DNA is potentially mutagenic. Furthermore, 8-NO_2-G formed in the nucleotide pool may trigger oxidative stress, may be incorporated into DNA and RNA, and may interfere with various important enzymes that use guanine nucleotides as substrates. Thus, 8-NO_2-G is not only a marker of RNS-induced nitrative DNA damage, but also may play roles in diverse pathophysiological conditions, such as inflammation, neurodegenerative and cardiovascular diseases, and cancer.

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ABBREVIATIONS

8-amino-G, 8-Aminoguanine; BSA, bovine serum albumin; dGuo, 2′-deoxyguanosine; GFP–SeV, Sendai virus fused to a green fluorescent protein gene; Guo, guanosine; HPLC, high-performance liquid chromatography; iNOS, inducible type of nitric oxide synthase; LPS, lipopolysaccharide; MPO, myeloperoxidase; MS, mass spectrometry; 8-NO_2-dGuo, 8-nitro-2′-deoxyguanosine; 8-NO_2-G, 8-nitroguanine; 8-NO_2-Guo, 8-nitroguanosine; nox-dGuo, 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine; 8-NO_2-X, 8-nitroxanthine; OV, *Opisthorchis viverrini*; 8-oxo-G, 8-oxo-7,8-dihydroguanine; 8-oxo-Guo, 8-oxo-7,8-dihydroguanosine; PMA, β-phorbol myristate acetate; RNS, reactive nitrogen species; SIN-1, 3-morpholinosydnonimine.

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Forum Review

Oxidative and Nitrative DNA Damage as Biomarker for Carcinogenesis with Special Reference to Inflammation

SHOSUKE KAWANISHI* and YUSUKE HIRAKU

ABSTRACT

Reactive oxygen and nitrogen species are known to participate in a wide variety of human diseases. Oxidative DNA damage is involved in chemical carcinogenesis and aging. Monocyclic chemicals induce mainly oxidative DNA damage, whereas polycyclic chemicals can induce oxidative DNA damage in addition to DNA adduct formation. Recently, chronic infection and inflammation have been recognized as important factors for carcinogenesis. Nitrative DNA damage as well as oxidative DNA damage is induced in relation to inflammation-related carcinogenesis. The authors examined the formation of 8-nitroguanine, a nitrative DNA lesion, in humans and animals under inflammatory conditions. An immunofluorescence labeling study demonstrated that 8-nitroguanine was strongly formed in gastric gland epithelial cells in gastritis patients with *H. pylori* infection, in hepatocytes in patients with hepatitis C, and in oral epithelium of patients with oral lichen planus. 8-Nitroguanine was also formed in colonic epithelial cells of model mice of inflammatory bowel diseases and patients with ulcerative colitis. Interestingly, 8-nitroguanine was formed at the sites of carcinogenesis regardless of etiology. Therefore, 8-nitroguanine could be used as a potential biomarker to evaluate the risk of inflammation-related carcinogenesis. *Antioxid. Redox Signal.* 8, 1047–1058.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) and reactive nitrogen species (RNS) are involved in a wide variety of human diseases, including cancer. Oxidative and nitrative stress refers to the situation of a serious imbalance of production of these reactive species and antioxidant defense system (22). ROS and RNS are capable of causing damage to various cellular constituents, such as nucleic acids, proteins, and lipids, leading to carcinogenesis, aging, and many other diseases. ROS are generated from multiple sources, including inflammatory cells, carcinogenic chemicals and their metabolites, and electron transport chain in mitochondria (Fig. 1). On the other hand, nitric oxide (NO) is generated specifically during inflammation via inducible nitric oxide synthase (iNOS) in inflammatory and epithelial cells (Fig. 1). Recent studies have provided evidence that inflammation is associated with carcinogenesis (14). Inflammation can be induced by chronic infection with various infectious agents and other physical, chemical and immunological factors (14, 77).

ROS and RNS are considered to play important roles in carcinogenesis through oxidative and nitrative DNA damage (34, 77). Guanine is most easily oxidized among the four DNA bases, because the oxidation potential of guanine is lower than the other three DNA bases, adenine, cytosine, and thymine (10, 108). ROS can induce the formation of oxidative DNA lesions, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (10, 20, 46, 119). 8-OxodG is considered to be a mutagenic DNA lesion. It was reported that misincorporation of adenine occurs opposite 8-oxodG during DNA synthesis, leading to G → T transversions (8, 104). The mutational spectra induced by other oxidative DNA lesions have been investigated. 2,5-Diamino-4H-imidazol-4-one (Iz) and 2,2,4-triamino-5-(2H)-oxazolone (Oz) can be generated by oxidation of guanine and 8-oxodG (10). Similarly to 8-oxodG, Oz induced G → T transversions (24), whereas Iz induced G → C transversions (54, 71).

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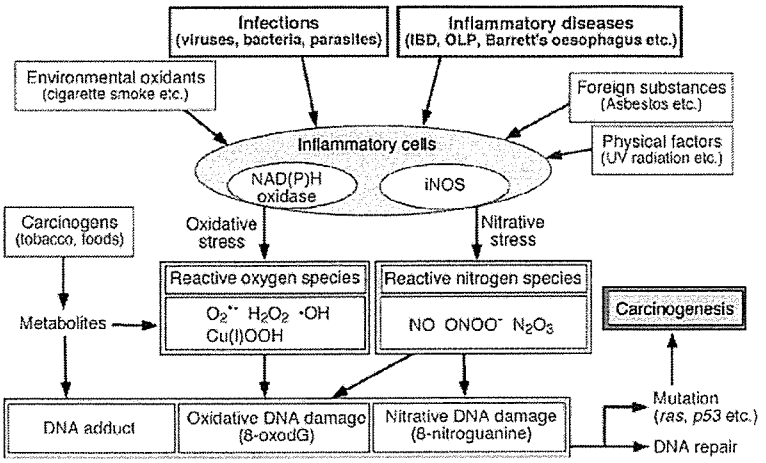

FIG. 1. Proposed mechanisms of oxidative and nitrative DNA damage leading to carcinogenesis induced by various environmental factors.

Excess NO production from inflammatory cells plays a critical role in an enormous variety of pathological processes, including cancer (34, 77). NO reacts with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$), a highly reactive species causing nitrative and oxidative DNA damage. $ONOO^-$ can mediate the formation of 8-oxodG (41) and 8-nitroguanine, a marker of nitrative DNA damage (122). Akaike *et al.* have demonstrated the 8-nitroguanine is formed via NO production associated with inflammation in mouse with viral pneumonia (2). 8-Nitroguanine is considered to be not only a marker of inflammation but also a potential mutagenic DNA lesion, leading to carcinogenesis. 8-Nitroguanine formed in DNA is chemically unstable, and thus can be spontaneously released, resulting in the formation of an apurinic site (122). The apurinic site can form a pair with adenine during DNA synthesis, leading to G → T transversions (59) (Fig. 2). Recently, it has been reported that adenine is preferentially incorporated opposite 8-nitroguanine during DNA synthesis, suggesting that G → T transversions can also occur via this mechanism (112). In the $ONOO^-$-treated supF shuttle vector plasmid, which was then replicated in *Escherichia coli*, the majority of mutations occurred at G:C base pairs, predominantly involving G → T transversions (43, 53). Therefore, 8-oxodG and 8-nitroguanine are potentially mutagenic lesions leading to carcinogenesis. Especially, we have proposed the possibility that 8-nitroguanine is a potential biomarker to evaluate the risk of inflammation-related carcinogenesis. Here we discuss the role of oxidative and nitrative DNA dam-

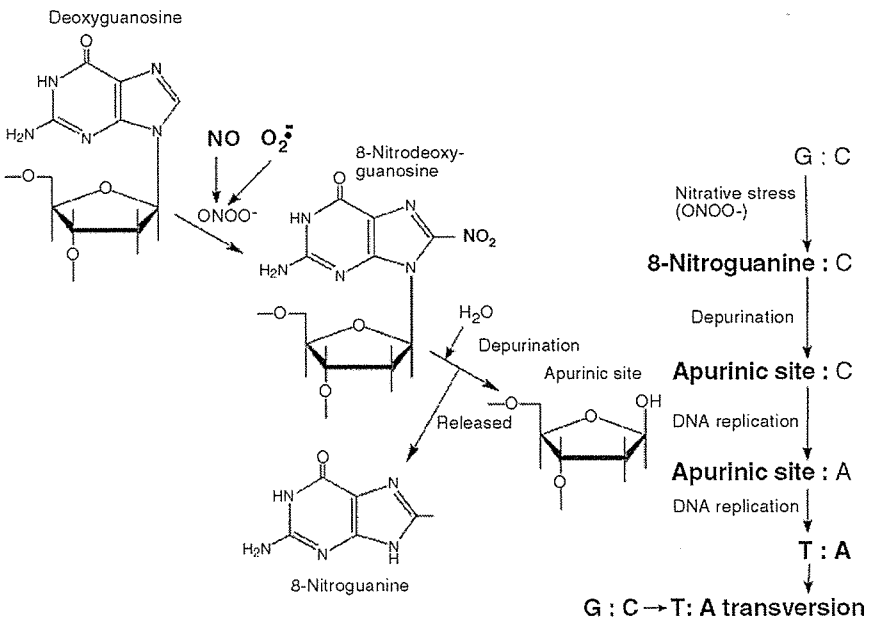

FIG. 2. Proposed mechanism of mutation mediated by 8-nitroguanine formation.

age in carcinogenesis caused by various environmental factors and inflammation.

OXIDATIVE DNA DAMAGE IN RELATION TO CHEMICAL CARCINOGENESIS AND AGING

In 1986, we discovered that the carcinogenic metal, chromium (VI), induced oxidative DNA damage in the presence of hydrogen peroxide (H_2O_2) (49). On the basis of this finding, we have proposed the hypothesis that metal carcinogenesis involves endogenous ROS generation. Other metal compounds, such as cobalt (120), nickel (50), and ferric nitrilotriacetate [Fe(III)-NTA] (40), also directly caused oxidative DNA damage in the presence of H_2O_2. Although the carcinogenicity of copper and iron has not been clear, a recent epidemiological study has demonstrated that the high intakes of copper and iron are associated with the increased risk of colorectal carcinogenesis (103). These findings regarding these metals suggest that free radicals play an important role in carcinogenesis. We demonstrated that copper (II) plus H_2O_2 induced DNA damage at thymine and guanine by generating ROS (121). Copper is an essential component of chromatin (16, 97). Copper accumulates in the liver of Long-Evans Cinnamon rats that spontaneously develop hepatocellular carcinomas (58). A case-cohort study showed a U-shaped relationship between plasma copper levels and risk of breast cancer (82). Iron is the most abundant transition metal ion in the human body. High body stores of iron may increase the risk of cancer in humans (109). Furthermore, renal cell carcinoma was observed in Fe(III)-NTA-treated rats (18). Fe(III)-NTA induced the formation of oxidative DNA lesions including 8-oxodG formation *in vivo* (1, 25). Genetic alterations in the *p15* and *p16* tumor suppressor genes were found in rats treated with Fe(III)-NTA (114).

Carcinogenic chemicals can induce various types of DNA damage including DNA adduct formation and oxidative DNA damage. We found that endogenous metals, particularly copper and iron, catalyzed ROS generation from various organic carcinogens and their metabolites, resulting in oxidative DNA damage (45). Table 1 shows the mutagenicity of various carcinogens and their potentials to induce DNA adduct formation and oxidative DNA damage. Although in 1976, the Ames test was believed to detect approximately 90% of carcinogens, a number of nonmutagenic carcinogens have been detected, and the concordance between this test and the carcinogenicity decreased to approximately 60%. There is a tendency that Ames test-negative carcinogens mainly cause oxidative DNA damage, whereas Ames test-positive carcinogens induce the formation of DNA adducts. Mutagenic carcinogens, such as polynuclear aromatic hydrocarbons [benzo[*a*]pyrene (76) and benz[*a*]anthracene (101, 102)] form DNA adducts predominantly, although these carcinogens are capable of causing oxidative DNA damage. Certain metabolites of benzo[*a*]pyrene (76) and benz[*a*]anthracene (102) induced double-base DNA damage consisting of piperidine-labile and formamidopyrimidine-DNA glycosylase (Fpg protein)-sensitive lesions at the 5′-ACG-3′ sequence (damaged bases are underlined) complementary to a hotspot of the *p53* tumor suppressor gene (Fig. 3). Aromatic nitro and amino compounds [4-aminobiphenyl (69) and heterocyclic amines (66–68)] also caused oxidative DNA damage to some extent. On the other hand, mononuclear compounds, such as benzene metabolites (26, 27, 47, 78), pentachlorophenol (70), *p*-

TABLE 1. DNA ADDUCT FORMATION AND OXIDATIVE DNA DAMAGE INDUCED BY CARCINOGENIC CHEMICALS

		DNA damage		*Ames*	
		Adduct	*Oxidation*	*test*	*References*
Aromatic hydrocarbons					
Mononuclear	Benzene	+	+++	−	26, 27, 78
"	Pentachlorophenol	+	+++	−	70
"	*p*-Dichlorobenzene	−	+++	−	79
"	Caffeic acid	−	+++	−	39
Polynuclear	Benzo[*a*]pyrene	+++	+	+	76
"	Benz[*a*]anthracene	+++	+	+	101, 102
Aromatic nitro and amino compounds					
Mononuclear	*o*-Toluidine	−	+++	−	73
"	*o*-Anisidine	−	+++	−	75
"	Nitrobenzene	−	+++	−	74
Polynuclear	4-Aminobiphenyl	+++	+	+	69
"	MeIQx and IQ	+++	+	+	67, 68
"	PhIP	+++	+	+	66
Others					
	2-Nitropropane	+	+++	±	96
	Benzoyl peroxide	−	+++	−	52
	Homogentisic acid	−	+++	−	29
	Aminoacetone	−	+++	−	28
	Estrogens	−	+++	−	30

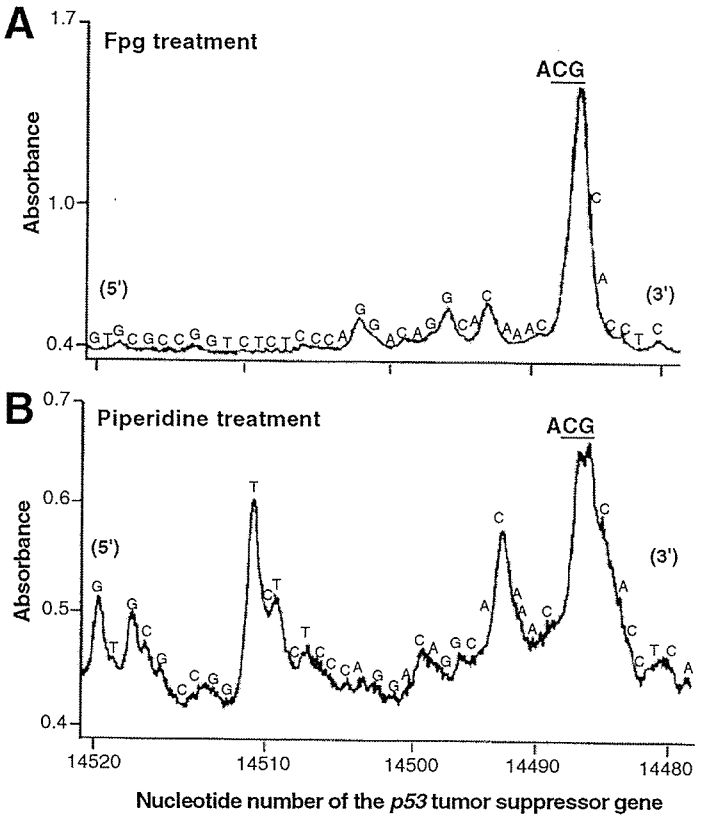

FIG. 3. Site specificity of DNA damage induced by a metabolite of benz[*a*]anthracene, benz[*a*]anthracene–3,4-dihydrodiol. The reaction mixture contained ^{32}P-5′-end-labeled DNA fragment of the *p53* tumor suppressor gene, 20 μ*M*/base calf thymus DNA, 20 μ*M* benz[*a*]anthracene–3,4-dihydrodiol, 100 μ*M* NADH, and 20 μ*M* $CuCl_2$ in 10 m*M* sodium phosphate buffer (pH 7.8) containing 5 μ*M* DTPA. The reaction mixtures were incubated at 37°C for 1 h, followed by the treatment with 6 units of Fpg protein at 37°C for 2 h (**A**) or 10%(v/v) piperidine at 90°C for 20 min (**B**). The treated DNA was electrophoresed on an 8% polyacrylamide/8 *M* urea gel. The autoradiogram was obtained by exposing X-ray film to the gel and then analyzed with a laser densitometer. (Adapted from Reference 102).

dichlorobenzene (79), *o*-toluidine (73), *o*-anisidine (75), nitrobenzene (74), caffeic acid (39), homogentisic acid (a tyrosine metabolite) (29), and aminoacetone (a threonine metabolite) (28) induced oxidative DNA damage predominantly, and thus, these compounds appear to express their carcinogenicity through oxidative DNA damage. In addition, certain metabolites of 2-nitropropane, an aliphatic amino compound, induced oxidative DNA damage in the presence of endogenous metals (96). Benzoyl peroxide plus Cu(I) caused DNA damage specifically at 5′-G of GG and GGG sequences in double-stranded DNA (52). This site specificity is explained by the *ab initio* molecular orbital calculation revealing that a large part of the highest occupied molecular orbital (HOMO) is distributed on the 5′-site guanine of GG sequences in double-helical DNA, and thus, this guanine is easily oxidized (110). Estrogen metabolites (catechol estrogens) induced oxidative DNA damage in the presence of Cu(II) at extremely low concentrations (30). Recently, we demonstrated that the addition of a histone peptide containing a metal-binding site enhanced oxidative DNA damage induced by H_2O_2 plus Cu(II) (63). These findings suggest that metal-mediated oxidative DNA damage contributes to carcinogenesis induced by various environmental chemicals.

In addition to carcinogenesis, oxidative DNA damage contributes to aging. We have reported that in cultured cells exposed to UVA radiation, oxidative stress induced the acceleration of telomere shortening, which has been considered to play the important role in aging (80). UVA irradiation with an endogenous photosensitizer riboflavin efficiently induced 8-oxodG formation at consecutive guanines in the ^{32}P-5′-end-labeled DNA fragments containing telomeric sequence (5′-TTAGGG-3′). Therefore, site-specific oxidative DNA damage appears to contribute to aging (51).

DNA DAMAGE IN RELATION TO INFLAMMATION-RELATED CARCINOGENESIS

Today, experimental and epidemiological evidence indicates that a variety of infectious agents constitute one of the main causes of cancer (14, 35). The International Agency for Research on Cancer (IARC) has estimated that approximately 18% of cancer cases worldwide is attributable to infectious diseases (35). The burden of cancer caused by infectious agents is shown in Table 2. Viruses are principal infectious agents, and bacterial and parasitic infections contribute to carcinogenesis to a lesser extent. Inflammation can be induced by chronic infection and many other physical, chemical, and immunological factors (14, 77). It has been hypothesized that many malignancies arise from areas of infection and inflammation (4, 14).

ROS and RNS play a key role in inflammation-mediated carcinogenesis. We demonstrated that carcinogenic nickel compounds induced oxidative DNA damage in rat lungs

TABLE 2. THE BURDEN OF CANCER CAUSED BY INFECTIOUS AGENTS WORLDWIDE

Infectious agent	*IARC classification**	*Cancer site*	*Number of cancer cases*	*% of cancer cases worldwide*
Bacterial infection				
H. pylori	1	Stomach	490,000	5.4
Viral infection				
HPV	1, 2A	Cervix and other sites	550,000	6.1
HBV, HCV	1	Liver	390,000	4.3
EBV	1	Lymphoma		
		Nasopharyngeal carcinoma	99,000	1.1
HHV-8	2A	Kaposi sarcoma	54,000	0.6
HTLV-1	1	Leukemia	9,000	0.1
Parasitic infection				
Schistosoma haematobium	1	Bladder	2,700	0.1
Liver flukes				
Opisthorchis viverrini	1	Cholangiocarcinoma	800	
Clonorchis sinensis	2A			
		Total infection-related cancers	1,600,000	17.7
		Total cancers in 1995	9,000,000	100

Adapted from 2003 IARC "World Cancer Report" (35).
*Group 1, carcinogenic to humans; Group 2A, probably carcinogenic to humans.

through inflammation (48). Akaike *et al.* have demonstrated that 8-nitroguanine is formed in association with inflammation in mice with viral infection (2). In relation to inflammation-related carcinogenesis, we examined the formation of 8-nitroguanine in addition to 8-oxodG using biopsy samples obtained from patients with inflammatory diseases and animals under inflammatory conditions. To examine the distribution of 8-nitroguanine, we produced highly sensitive and specific anti-8-nitroguanine antibody without cross reaction, as shown in Fig. 4 (86). Here we discuss the role of nitrative and oxidative DNA damage in carcinogenesis arising from various inflammatory conditions.

Oxidative and nitrative DNA damage induced by infectious agents

Liver fluke infection and cholangiocarcinoma. Infection with the liver fluke *Opisthorchis viverrini* (OV) is a major risk factor of cholangiocarcinoma, especially in the northeastern region of Thailand (23, 38) and has been evaluated to be carcinogenic to humans (Group 1) (38). Infection with this parasite can be repeatedly caused by eating raw or undercooked fish containing the infective stage of the fluke. A majority (approximately 70%) of OV-induced cholangiocarcinoma occurs in the intrahepatic bile ducts, and the remainder occurs in the extrahepatic duct (118). However, the mechanism by which OV induces cholangiocarcinoma remains to be understood.

We investigated DNA damage in the liver of hamsters with single and repeated OV infection as a model of inflammation-related carcinogenesis in humans. We were first in reporting that 8-nitroguanine is formed in relation to inflammation-related carcinogenesis using this animal model (90). Double immunofluorescence staining revealed that 8-oxodG and 8-nitroguanine were formed in inflammatory cells and epithelium of bile ducts (86, 87). The immunoreactivities of 8-oxodG and 8-nitroguanine in inflammatory cells were most prominently observed on days 21 and 30, respectively (86). It is noteworthy that these DNA lesions still remained in the epithelium of bile ducts on day 180. The formation of 8-nitroguanine and 8-oxodG increased in the epithelium of bile ducts in the order of three-time infection > two-time infection > single infection. This may be explained by the fact that repeated infection increased iNOS expression in the epithelium of bile ducts in the same order (87). Proliferating cell nuclear antigen (PCNA), which functions as a cofactor for DNA polymerase δ, is associated with DNA replication and long-patch base excision repair (20, 99). In our study, PCNA accumulated in the epithelium of bile ducts after repeated OV infection, supporting the hypothesis that cell proliferation was promoted by inflammation-mediated DNA damage (87). Recently, we reported that OV antigen induces inflammatory response, including iNOS expression, through Toll-like receptor (TLR)-2-mediated pathway (89). We found that the formation of 8-oxodG and 8-nitroguanine occurred to a much greater extent in cancerous tissue than in noncancerous tissue in intrahepatic cholangiocarcinoma patients, and that these DNA lesions contribute to tumor progression (88). In conclusion, more frequent OV infection can induce the iNOS expression in the epithelium of bile ducts and subsequently cause nitrative and oxidative damage to nucleic acids, which may participate in every step of cholangiocarcinoma development, including initiation, promotion and progression.

Helicobacter pylori *infection and gastric cancer.* The presence of the Gram-negative bacterium, *Helicobacter*

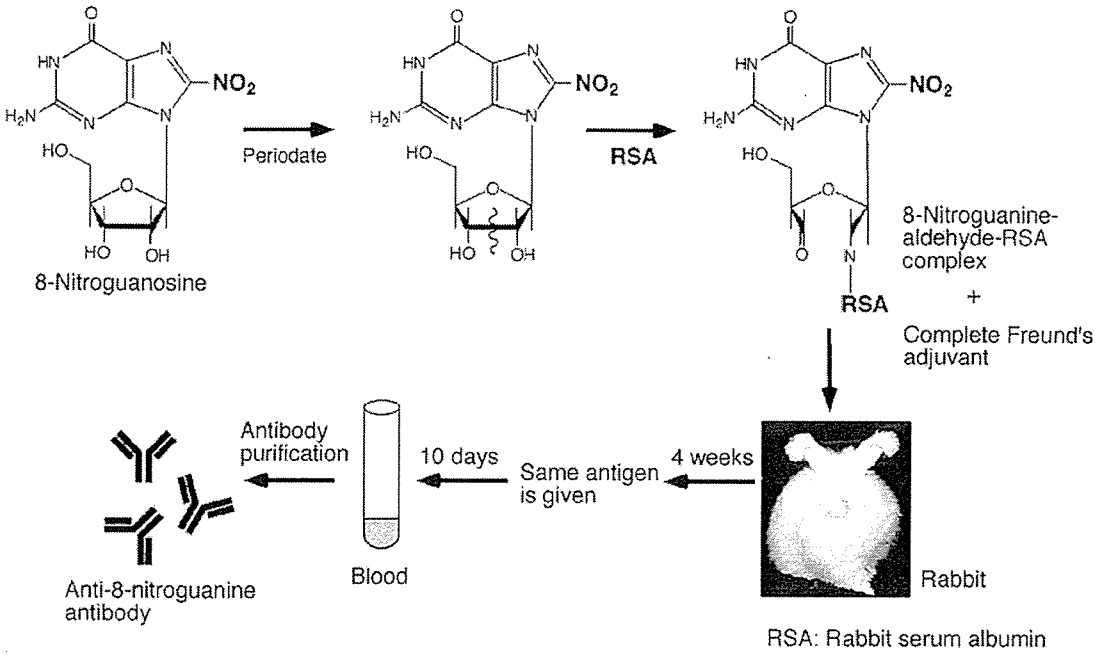

FIG. 4. Production of polyclonal anti-8-nitroguanine antibody. 8-Nitroguanosine was incubated with sodium metaperiodate and then conjugated with rabbit serum albumin (RSA). The 8-nitroguanine-aldehyde–RSA conjugate mixed with Freund's complete adjuvant was injected in rabbit intracutaneously. After 4 weeks, the same antigen was given and the blood was taken 10 days later. We purified the anti-8-nitroguanine antibody by affinity chromatography. Specificity of the purified antibody was examined by a dot immunobinding assay and absorption test (86).

pylori (H. pylori) is associated with not only chronic atrophic gastritis and peptic ulcer but also gastric adenocarcinoma and non-Hodgkin's lymphoma [mucosa-associated lymphoid tissue (MALT) lymphoma] (83). *H. pylori* infection has been evaluated to be carcinogenic to humans (Group 1) (36). The mechanisms by which *H. pylori* infection causes gastric cancer have been investigated. Lipopolysaccharide (LPS), which is a component of Gram-negative bacteria including *H. pylori*, is a ligand of TLR4. TLR4 is involved in activation of the transcription factor nuclear factor-κB (NF-κB) (61). Another study has revealed that TLR2 and TLR5, but not TLR4, are required for *H. pylori*-induced NF-κB activation by epithelial cells (107). A recent report has shown that NF-κB functions as a tumor promoter in inflammation-associated cancer (85). NF-κB is involved in regulation of iNOS expression (111). Thus, it is necessary to examine whether *H. pylori*-mediated iNOS expression leads to 8-nitroguanine formation in gastric epithelial cells and this DNA lesion contributes to carcinogenesis.

We performed a double immunofluorescence labeling study and demonstrated that the intense immunoreactivities of 8-nitroguanine and 8-oxodG were observed both in gastric gland epithelial cells and inflammatory cells in patients with *H. pylori* infection (60). On the other hand, in gastritis patients without *H. pylori* infection, these immunoreactivities were observed in inflammatory cells but not in gastric gland epithelial cells. This finding is supported by the previous study demonstrating that the formation of 8-oxodG is increased in the gastric epithelium of *H. pylori*-infected patients (3, 84). It has been reported that the expression of iNOS mRNA and protein was significantly increased in *H. pylori*-positive gastritis compared to *H. pylori*-negative gastritis (21). Specific cytokines are considered to participate in *H. pylori*–induced iNOS expression in gastric mucosa. Cag-positive *H. pylori* strain induces an intense inflammatory responses including interleukin (IL)-8 production from epithelial cells and subsequent production of tumor necrosis factor (TNF-α) from inflammatory cells (83). These cytokines have been reported to participate in iNOS expression in gastric mucosa (57, 115). Collectively, the host immune response to *H. pylori* mediated by cytokines, resulting in iNOS expression, may lead to an increase in the accumulation of 8-nitroguanine and 8-oxodG in gastric epithelium.

Several studies have demonstrated that PCNA is an independent prognostic factor for gastric cancer in patients with *H. pylori* infection (99). In our study, the accumulation of PCNA was significantly higher in gastric gland epithelial cells in patients with *H. pylori* infection compared to those without infection (60). These results suggest that nitrative and oxidative DNA damage in gastric epithelial cells and their proliferation by *H. pylori* infection may lead to gastric carcinoma.

Hepatitis C virus infection and liver cancer.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma throughout the world (37, 93). HCV infection has been evaluated as a

Group 1 carcinogen by IARC (37). It is generally accepted that hepatocellular carcinoma arises through a multistep process of genetic alterations in hepatocytes during chronic hepatitis C (CHC) (7, 11, 33, 72). It has been demonstrated that 8-oxodG is accumulated in hepatocytes from patients with chronic viral hepatitis (55, 105). Several studies on patients with chronic HCV have shown that hepatic iron overload is attributable to liver injury and that iron depletion improved serum aminotransferase levels (42, 44) and normalized elevated hepatic 8-oxodG level (44). Therefore, iron overload may be involved in generation of reactive species to form mutagenic DNA lesions. Alternatively, HCV core protein is capable of inducing oxidative stress in cultured cells and animals (81), and this protein has been demonstrated to participate in hepatic carcinogenesis in transgenic mice (65). Moreover, a HCV-encoded nonstructural protein (NS3) has been reported to participate in generation of oxygen radicals from phagocytes via activation of NADPH oxidase (9, 116). However, the mechanism of HCV infection-induced hepatitis followed by hepatocarcinogenesis via DNA damage is still unclear.

We investigated DNA damage in liver biopsy specimens of patients with CHC and the effect of interferon treatment. The immunoreactivities of 8-nitroguanine and 8-oxodG were strongly detected in the liver from patients with CHC, but not in control livers (nonalcoholic fatty liver) (32). 8-Nitroguanine accumulation was found in not only infiltrating inflammatory cells but also hepatocytes particularly in the periportal area. The accumulation of 8-nitroguanine and 8-oxodG increased with inflammatory grade. iNOS expression was observed in the cytoplasm of hepatocytes and Kupffer cells in CHC patients. In the sustained virological responder group after interferon therapy, the accumulation of 8-nitroguanine and 8-oxodG in the liver was markedly decreased (32). Our results are consistent with the previous reports showing that the expression of iNOS in hepatocytes has been observed in patients with chronic hepatitis (62) and hepatocellular carcinoma (94). Taken together, these findings indicate that 8-nitroguanine is a useful biomarker to evaluate the severity of HCV-induced chronic inflammation leading to hepatocellular carcinoma. 8-Nitroguanine could also be used for the evaluation of the efficacy of CHC treatment.

Oxidative and nitrative DNA damage induced by noninfectious agents

Inflammatory bowel disease and colon cancer. Ulcerative colitis and Crohn's disease, which are referred to as inflammatory bowel diseases (IBD), are well known as chronic inflammatory diseases in lower bowel. These diseases lead to long-term impairment of intestinal structure and function (6, 91). Epidemiological studies have shown that the incidence of colorectal cancer in IBD is greater than the expected incidence in the general population (13, 19, 56). Although the precise mechanisms of the pathogenesis of IBD have not been clarified, a large number of immunological abnormalities has been noted in patients with IBD (6, 91). Inflammation occurs as a result of either excessive functions of effector T cells, such as T helper type 1 (Th1) and 2 (Th2) cells or deficient function of regulatory T cells.

On the basis of the hypothesis that the imbalance of helper and regulatory T cell functions plays the key role in IBD pathogenesis, we prepared a mouse model of IBD. For induction of IBD, C.B-17 SCID mice were injected intraperitoneally with purified $CD45RB^{high}CD4^{+}$ T cells (mainly consisting of Th1 and Th2 cells lacking regulatory T cells) as described previously (92). This IBD mouse model showed that the body weight increased with aging to a lesser extent than nontreated controls and that the intestine was shortened. Pathological findings of this mouse model, which showed severe inflammation in colon tissues, were similar to IBD patients. Double immunofluorescence technique revealed that both 8-nitroguanine and 8-oxodG were formed mainly in epithelial cells of the IBD mouse model (17). When the tissue sections were pretreated with RNase, 8-nitroguanine immunoreactivity was more clearly observed in the nuclei of epithelial cells, suggesting that 8-nitroguanine is formed mainly in genomic DNA. The formation of 8-nitroguanine in the nuclei was confirmed by electron microscopic immunohistochemistry. iNOS, PCNA, and p53 proteins were also expressed in the colon epithelium. We also demonstrated that 8-nitroguanine was formed in colon epithelium of patients with ulcerative colitis (unpublished data). Relevantly, several studies have shown that iNOS is expressed in the epithelial cells in colitis patients (31, 106, 119). In noncancerous colon tissues from patients with ulcerative colitis, iNOS protein levels were positively correlated with p53 serine 15 phosphorylation levels (31). These results suggest that nitrative DNA damage, as well as oxidative DNA damage, participates in colon carcinogenesis in patients with IBD.

Oral lichen planus and oral cancer. Oral lichen planus (OLP) is a chronic inflammatory mucosal disease (100). Several pathological features indicate that OLP is immunologically-mediated inflammatory response, including an intense, band-like infiltrate of predominantly T-lymphocytes subjacent to epithelium. The basal epithelial cells are the target for immune destruction by cytotoxic T lymphocytes (15, 117). The most important complication of OLP is development of oral squamous cell carcinoma (OSCC) (64, 95). However, DNA damage associated with OLP and OSCC has not been investigated.

We demonstrated that the accumulation of 8-nitroguanine and 8-oxodG was apparently observed in oral epithelium of biopsy specimens of patients with OLP and OSCC, whereas no immunoreactivity was observed in normal oral mucosa (Table 3) (12). Colocalization of 8-nitroguanine and iNOS was found in oral epithelium of OLP and OSCC patients. Immunoreactivity of 3-nitrotyrosine, which is formed by protein tyrosine nitration and considered to be a biochemical marker for inflammation, was also observed in oral epithelial cells. Accumulation of p53 was more strongly observed in oral epithelium in OSCC than OLP, whereas there was no p53 accumulation in normal oral mucosa (Table 3). Our findings demonstrate that iNOS-dependent DNA damage in OLP may lead to p53 accumulation in not only OLP but also OSCC. It is concluded that the formation of 8-nitroguanine and 8-oxodG may contribute to development of oral cancer from OLP.

TABLE 3. CORRELATION OF IMMUNOREACTIVITIES OF 8-NITROGUANINE, 8-OXODG, INOS, AND P53 IN THE BASAL LAYER OF ORAL EPITHELIUM IN OLP PATIENTS AND NORMAL MUCOSA

Immuno-reactivity grading	*8-nitroguanine*			*8-oxodG*			*iNOS*			*P53*		
	NM	*OLP*	*OSCC*	*NM*	*OLP*	*OSCC*	*NM*	*OLP*	*OSCC*	*NM*	*OLP*	*OSCC*
−	15	0	0	15	0	0	15	3	0	15	3	0
+	0	1	0	0	1	1	0	4	1	0	7	1
++	0	6	2	0	6	2	0	3	0	0	1	0
+++	0	4	1	0	4	0	0	1	2	0	0	2
P	0.00001			0.00001			0.00127			0.00038		

NM = normal mucosa (n = 15); OLP = oral lichen planus patients (n = 11); OSCC = oral squamous cell carcinoma patients (n = 3). The following scores were assigned to each specimen according to the degree of staining: −, negative; +, <25%; ++, 25%–50%; and +++, >50% of the cells in tissue sections. The significant difference between NM and OLP was analyzed by Chi-square test.

CONCLUSION

We have investigated the mechanisms of oxidative and nitrative DNA damage induced by various carcinogenic chemicals and inflammatory conditions. We have demonstrated that monocyclic chemicals and many other chemicals induce oxidative DNA damage rather than DNA adduct formation. On the other hand, polycyclic chemicals can induce oxidative DNA damage to some extent in addition to DNA adduct formation. Therefore, the contribution of these carcinogens to each type of DNA lesion seems to be dependent on their chemical structures and properties.

In relation to inflammation-related carcinogenesis, we examined the formation of 8-nitroguanine and 8-oxodG in human samples and animals. It is noteworthy that DNA damage was specifically induced at sites of carcinogenesis under various inflammatory conditions. In human samples, 8-nitroguanine formation was observed in gastric gland epithelial cells of patients with *H. pylori* infection (60) and in hepatocytes of patients with chronic hepatitis C (32). 8-Nitroguanine was also formed in oral epithelium of OLP and OSCC patients (12). Moreover, in hamsters infected with the liver fluke OV causing cholangiocarcinoma, 8-nitroguanine formation was induced in bile duct epithelium (87). 8-Nitroguanine formation was also found in colonic gland epithelial cells of mouse model of IBD (17). Therefore, 8-nitroguanine could be used as a potential biomarker to evaluate the risk of inflammation-related carcinogenesis. Recently, 8-nitroguanosine has been reported to be a highly redox-active molecule that strongly stimulates $O_2^{\cdot-}$ generation from NADPH-dependent reductases (98). 8-Nitroguanine may be a cofactor for redox reaction and cell signaling implicated in diverse physiological and pathological events (123). More importantly, experimental evidence has suggested that 8-nitroguanine is a mutagenic DNA lesion, which preferentially leads to G → T transversions (112, 122), in addition to 8-oxodG (8, 104). Indeed, G → T transversions have been observed *in vivo* in the *ras* gene (5) and the *p53* tumor suppressor gene in lung and liver cancer (33, 113). These findings imply that DNA damage mediated by ROS and RNS may participate in carcinogenesis via activation of protooncogenes and inactivation of tumor suppressor genes. In conclusion, oxidative and nitrative DNA damage could be promising biomarkers to evaluate the risk of carcinogenesis induced by a wide variety of chemicals and inflammatory conditions.

ABBREVIATIONS

CHC, chronic hepatitis C; Fe(III)-NTA, ferric nitrilotriacetate; HCV, hepatitis C virus; H_2O_2, hydrogen peroxide; HOMO, highest occupied molecular orbital; IARC, International Agency for Research on Cancer; IBD, inflammatory bowel diseases; IL, interleukin; iNOS, inducible nitric oxide synthase; Iz, 2,5-diamino-4H-imidazol-4-one; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; OLP, oral lichen planus; $ONOO^-$, peroxynitrite; OSCC, oral squamous cell carcinoma; OV, *Opisthorchis viverrini*; Oz, 2,2,4-triamino-5-(2H)-oxozolone; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PCNA, proliferating cell nuclear antigen; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSA, rabbit serum albumin; Th, T helper; TLR, Toll-like receptor; TNF, tumor necrosis factor.

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