MINI-REVIEW

Reactive Oxygen and Nitrogen Oxide Species-induced Stress, a Major Intrinsic Factor Involved in Carcinogenic Processes and a Possible Target for Cancer Prevention

Dai Nakae1*, Takashi Umemura2, Yuji Kurokawa3

Abstract

Reactive oxygen and nitrogen oxide species and their inducing stress are involved in a variety of physiological and pathological phenomena in aerobes, including humans. For multistage carcinogenic processes, reactive oxygen and nitrogen oxide species-induced stress (RONOSS) serves as a major intrinsic factor and is involved in every step. This means that free radicals, RONOSS and their inducing downstream events may be targets for cancer prevention. It is therefore of importance to elucidate the mechanisms underlying the participation of RONOSS in carcinogenesis and to apply the obtained results for establishment of strategies to control cancer development. Despite the large body of accumulated knowledge due to worldwide efforts dealing with this research field, there still remain numerous uncertainties. In this mini-review, we introduce two examples of such efforts, one concerning a renal carcinogen KBrO3 and the other dealing with hepatocarcinogenesis caused by a choline-deficient, l-amino acid-defined diet, in order to give some idea about the current understanding of the roles of RONOSS in carcinogenesis.

Key Words: Reactive oxygen species - 8-hydroxydeoxyguanosine - potassium bromate - signal transduction abnormalities - choline-deficient-l-amino acid-defined diet - carcinogenesis

Introduction

Carcinogenesis involves a series of qualitatively different stages that occur as a result of the sequential accumulation of genetic and epigenetic lesions. While these lesions are induced mainly by exposure to extrinsic factors such as various substances present in the environment, intrinsic factors have also attracted attention for their participation in carcinogenic processes (Ames et al., 1995; Loeb, 1989; Pitot et al., 1991). Among such intrinsic factors, reactive oxygen and nitrogen oxide species-induced stress (RONOSS) have been shown to play significant roles through a wide variety of alterations including injury to subcellular components, mutations, abnormal gene regulation and signal transduction abnormalities (Deshpande and Irani, 2002; Epe, 2002; Jackson and Loeb, 2001; Kovacic and Jacinthe, 2001; Lala and Chakraborty, 2001). On the other hand, it has become well recognized that the presence of continuous stimulation, such as chronic inflammation, is crucially involved in many cases of development of cancers. Typical examples of factors causing such continuous stimulation include chronic viral hepatitis for hepatocellular carcinomas (Montalto et al., 2002), cholelithiasis and chronic inflammation due to varied infectious and non-infectious reasons for biliary tract cancers (Lazcano-Ponce et al., 2001; Tazuma and Kajiyama, 2001), chronic inflammation due to the infection of Helicobacter pylori for gastric cancers (Ebert et al., 2002; Peek and Blaser, 2002; Terry et al., 2002), and hormonal imbalance for mammary and genital cancers (Cavalieri and Rogan, 2002, Imagawa et al., 2002; Liao and Dickson, 2002). Such continuous stimulation may cause tumor-induction per se, but in most cases it serves to provide a “hypercarcinogenic state” which enhances carcinogenic processes (Shimizu et al., 1999 and 2000; Umeda and Hino,
RONOSS play significant roles in most of these continuous stimulation cases, and this is one of the most typical pathways for stress to be involved in neoplasia (Ambas et al., 1999; Deshpande and Irani, 2002; Lala and Chakraborty, 2001; Shacter and Weitzman, 2002). In this sense, free radicals, RONOSS and their downstream events are essential intrinsic factors and may thus be good targets for cancer prevention. It is then of importance to investigate concrete examples of their participation in carcinogenesis to draw conclusions for practical strategies to control cancers. In this mini-review, we introducing two models, one concerning the renal carcinogen KBrO₃ and the other dealing with hepatocarcinogenesis caused by a choline-deficient, L-amino acid-defined (CDAA) diet in the absence of any carcinogen exposure, in order to give some idea about the current understanding of the roles of RONOSS in carcinogenesis.

Possible Roles for an 8-Hydroxydeoxyguanosine (8-OHdG) Adduct in KBrO₃ Carcinogenesis

KBrO₃ has been classified as a “genotoxic” carcinogen based on positive mutagenicity in the Ames (Ishidate et al., 1984), chromosome aberration (Ishidate and Yoshioka, 1980) and micronuclear (Hayashi et al., 1988) tests, and positive carcinogenicity in rats after a 2-year oral administration with the standard long-term bioassay protocol (Kurokawa et al., 1982). We have shown that antioxidants, especially sulfhydryl compounds, are protective against the clastogenicity of KBrO₃, suggesting the participation of reactive oxygen species in its underlying mechanisms of action (Sai et al., 1992a). In fact, ribo- and deoxyribonucleosides of 8-hydroxyguanine induce sister chromatid exchange in human lymphocytes (Arashidani et al., 1998). We have, therefore, hypothesized that the oxidizing property of KBrO₃ is responsible for its “genotoxicity” and carcinogenicity.

Generation of Reactive Free Radicals by KBrO₃

We have analyzed reactive oxygen species caused by the interaction of KBrO₃ with cells from rat kidney by means of electron spin resonance spectrometry and revealed generation of 5,5-dimethyl-1-pyrroline-N-oxide-hydroxide, this being inhibited by singlet oxygen scavengers such as dimethylsulfoxide and ethanol. Superoxide dismutase, catalase and various hydroxyl radical scavengers, were not found to exert any significant inhibition. Furthermore, using 2,2,6,6-tetramethylpiperidine, a singlet oxygen-trapping agent, its oxide could be detected in mixtures of KBrO₃ with homogenates of kidney tissue. Together with our data from the chemiluminescence analysis, the results suggested that singlet oxygen is the most probable candidate for the reactive oxygen species generated by KBrO₃ (Sai et al., 1992b). On the other hand, it was recently reported that the reduction of KBrO₃ by sulfhydryl compounds such as glutathione (GSH) and cysteine (Cys) results in yields of bromine oxides and bromine radicals, which can effectively cause the guanine oxidation (Murata et al., 2001).

8-OHdG Formation by KBrO₃

It is well known that reactive free radicals attack cellular DNA and nucleotide pools to form various types of oxidized bases; e.g., 8-OHdG (Kasai et al., 1998), 5-hydroxydeoxyctydine (Feig et al., 1994) and 2-hydroxydeoxyadenosine (Kamiya and Kasai, 1995). Among these oxidized bases, 8-OHdG has especially received much attention because of the accumulated data indicating that it may play crucial roles in a chain of events from RONOSS to carcinogenesis (Kasai, 1997). 8-OHdG pairs with adenine as well as cytosine, subsequently yielding GC-to- TA transversions upon replication by DNA polymerases (Cheng et al., 1992; Shibutani et al., 1991). This mutation is considered to contribute to the activation of oncogenes and/or the inactivation of tumor suppressor genes, leading to carcinogenesis (LePage et al., 1995). In fact, it has been demonstrated that KBrO₃ has the potential to induce 8-OHdG formation in both in vitro and in vivo situations.

In vitro: The 8-OHdG levels were increased in renal proximal tubular cells isolated from rats using collagenase and incubated with KBrO₃ and in isolated rat renal nuclei incubated with a lipid-peroxidation system (Sai et al., 1994). In the presence of GSH, KBrO₃ also induces 8-OHdG modification in DNA of bacteriophage PM2 (Ballmaier and Epe, 1995), calf thymus DNA (Chipman et al., 1998; Persons and Chipman, 2000) and the human leukemia cell line HL-60 and its H₂O₂-resistant clone, HP100 (Murata et al., 2001). This 8-OHdG formation by KBrO₃ then indeed causes gene mutations, as shown at the HPRT locus of V79 Chinese hamster cells (Speit et al., 1999) and in p53 exon 7 of LLC-PK1 cells (Richter and Vamvakas, 1998). Additionally, depletion of GSH by buthionine sulfoximine exerts only a limited inhibitory effect on KBrO₃-induced 8-OHdG formation in DNA of human leukemia cell lines, suggesting an important role for Cys as well as GSH (Murata et al., 2001). It is thus indicated that KBrO₃ requires reduction systems, especially those featuring sulfhydryl groups provided by GSH and Cys, to exert its oxidizing properties leading to oxidative DNA damage. In the kidney, GSH is filtered through glomeruli into the lumen of renal tubules and then hydrolyzed by the brush-border enzymes to the constituent amino acids including Cys, which are reabsorbed in the proximal tubules. The location of the 8-OHdG formation by KBrO₃ may thus be in accordance with the target site of the carcinogen.

In vivo: A single administration of KBrO₃ to rats by gavage causes elevation of 8-OHdG levels in the kidney DNA, but not in liver DNA (Kasai et al., 1987). A single intraperitoneal injection of KBrO₃ also increases the 8-OHdG level in rat kidney DNA (Sai et al., 1991). In addition, such increase in the 8-OHdG level following a single administration of KBrO₃ is inhibited by co-treatment with various antioxidants; such as vitamin C (Sai et al., 1992c), resveratol, melatonin and vitamin E (Cadenas and Barja, 1999). While the doses of KBrO₃ used in these studies were
considerably higher than its carcinogenic dose, we have shown that the 8-OHdG levels in the kidney DNA, but not in liver DNA, significantly increase from the ends of weeks 1 and 4, respectively, in male and female rats continuously administered KBrO₃ at an concentration of 500 ppm in the drinking water, and that high 8-OHdG levels are maintained until the end of week 13 (Umemura et al., 1995 and 1998). A 13-week administration of KBrO₃ at 500 ppm in the drinking water is sufficient to induce renal cell tumors in male rats after a subsequent 91-week maintenance of animals on drinking water without any carcinogen (Kurokawa et al., 1990). However, another group claimed that a single intragastric administration of KBrO₃ to rats at a dose enough to elevate the 8-OHdG level fails to exert initiating activity in a renal carcinogenesis model (Kurata et al., 1992). It is, therefore, plausible that persistent increase of the 8-OHdG level is required for generation of gene mutations sufficient for carcinogenicity of KBrO₃.

**Repair Enzymes for 8-OHdG**

In *Escherichia coli*, three kinds of DNA glycosylases have been found to prevent mutagenesis caused by 8-OHdG. Fpg protein acts in the excision of 8-OHdG (Michaels et al., 1991). MutY protein, in contrast, excises adenine residues incorporated by DNA polymerases opposite 8-OHdG (Tajiri et al., 1991) and Mut T protein hydrolyses 8-OHdGTP from the pool of DNA precursors (Maki and Sekiguchi, 1992). Recently, a gene encoding a human repair glycosylase for 8-OHdG, hOGG1, was cloned (Rosenquist et al., 1997). It has been shown that the repair rate of 8-OHdG induced by KBrO₃ in a Chinese hamster ovary cell line is more rapid in hOGG1 transfectants than in the parental cells. The overexpression of OGG1, however, does not affect mutation frequencies of the gpr locus in ASS2 cells exposed to KBrO₃ (Hollenbach et al., 1999). At present, therefore, the relationship between the repair systems for 8-OHdG and KBrO₃-initiated carcinogenesis is still a matter of controversy, although the OGG1 deficient mouse might provide some information (Klungland et al., 1999).

**Hepatocarcinogenesis in Rats Fed a CDAA Diet and the Participation of RONOSS in Its Underlying Mechanisms**

Dietary choline deficiency with limited methionine availability in rats induces hepatocellular carcinomas in male Fischer 344 rats in the absence of any exposure to exogenous carcinogenic substances (Ghoshal and Farber, 1984; Locker et al., 1986; Newberne et al., 1982). The cancer incidence, however, is low at around 30-40% when using conventional choline-deficient diets, which makes it difficult to explore underlying carcinogenic mechanisms (Ghoshal and Farber, 1984; Locker et al., 1986; Newberne et al., 1982). To overcome this problem, we have produced a new diet deficient in choline and low in methionine and demonstrated strong hepatocarcinogenicity of this CDAA diet in male Fischer 344 rats (Nakae et al., 1990 and 1992). Using this diet, we have been investigating the mechanisms underlying “endogenous” carcinogenesis and exploring strategies for cancer chemoprevention (Nakae, 1999 and 2000).

**Characteristics of Hepatocarcinogenesis in Rats fed the CDAA diet**

When male Fischer 344 or Wistar rats are fed the CDAA diet continuously, hepatocellular carcinomas begin to develop at the end of week 40 and reach an incidence of almost 100% at the end of week 104 (Nakae, 1999; Nakae et al., 1992). The hepatocarcinogenesis proceeds with background induction of fatty liver (by day 2), apoptotic death and proliferation of hepatocytes (from day 3 and thereafter repeatedly continuing) and fibrosis (from week 4 and thereafter progressing) (Nakae, 1999). Fibrosis becomes frank cirrhosis by the end of week 30 (Nakae, 1999). In the presence of these non-tumor changes, foci of cellular alteration with positive glutathione S-transferase placental form (GST-P) phenotype are induced and grow (Nakae, 1999). The foci then progress to hepatocellular adenomas and are finally converted into hepatocellular carcinomas (Nakae, 1999). The histological sequence for the development of hepatocellular carcinoma in rats fed the CDAA diet thus closely resembles that seen in human cases especially those preceded by hepatitis C virus infection (Yao and Terrault, 2001). The liver damage induced in rats by the CDAA diet feeding and chronically present during the hepatocarcinogenic processes may serve as a continuous stimulation to cause a “hypercarcinogenic state”, as does chronic hepatitis induced in humans by hepatitis C virus infection.

**Involvement of RONOSS in Hepatocarcinogenesis in Rats fed the CDAA diet**

When male Fischer 344 or Wistar rats are fed the CDAA diet, hydrogen peroxide is over-produced in hepatocyte mitochondria 3 days after the commencement (Hensley et al., 2000), suggesting the induction of RONOSS in the livers from a very early time-point. The hepatocyte nuclear DNA is oxidatively injured, and the levels of 8-OHdG are significantly elevated by the end of the first day of the feeding and accumulate thereafter (Yoshiji et al., 1992). Other sub-hepatocellular components are then oxidatively injured, detected as the significantly increased levels of 2-thiobarbituric acid-reacting substances (TBARS) at the end of day 3, the levels increasing continuously thereafter (Yoshiji et al., 1992). Whereas the exact nature of the TBARS is not as yet identified, byproducts of peroxidation of lipids and proteins are suggested to contribute (Nakae, 1999). In fact, α-6 lipid hydroperoxide-mediated DNA adducts are generated in the hepatocytes of rats fed the CDAA diet for 3 days (Kawai et al., 2002). These findings indicate that RONOSS is indeed induced in hepatocytes of rats from the very beginning of the CDAA diet feeding and that the stress continues as long as the diet is fed. Then the next query must be whether RONOSS is biologically significant for “endogenous” carcinogenesis. The time-course of the accumulation of TBARS significantly
correlates with those of hepatocyte apoptosis and proliferation (Nakae, 1999). Furthermore, it appears that 8-OHdG formation and TBARS generation are critically involved in the induction and growth of putatively preneoplastic, GST-P-positive, hepatocyte focal lesions (Kobayashi et al., 1998; Nakae et al., 1994). In addition, various chemicals modify the extent of apoptosis and proliferation of hepatocytes and hepatocarcinogenesis itself in rats fed the CDAA diet in association with the concurrent modification of RONOSS-related changes (Denda et al., 2002; Kobayashi et al., 1998; Nakae, 1999 and 2000; Nakae et al., 1994 and 1998). It is thus strongly suggested that RONOSS induced by the continuous feeding of the CDAA diet is causally involved in the induction of a “hypercarcinogenic state” due to continuous liver damage and, in turn, progress of “endogenous” hepatocarcinogenesis in rats. While the underlying mechanisms remain largely obscure, work has revealed the generation of reactive nitrogen oxide species via the induction of inducible nitric oxide synthase, activation of several transcription factors including nuclear factor-κB and activator protein-1, induction and activation of inducible cyclo-oxygenase (Denda et al., 2002; Nakae et al., 1998), and the sequential and accumulating overexpression of cell cycle regulating protooncogenes (Tsujuchi et al., 1995) and various cytokines (Nakae, 1999). It is, therefore, indicated that RONOSS causes a variety of signal transduction alterations, and these may provide a main clue as to the causative involvement of stress in “endogenous” hepatocarcinogenesis in rats fed the CDAA diet.

Conclusions

Multi-stage carcinogenic mechanisms are mediated by both extrinsic and intrinsic factors. It is thus necessary to elucidate how these act alone and in combination to participate in neoplastic processes. Assuming the importance of RONOSS as a major intrinsic factor, in this context, the precise mechanisms underlying the influence of RONOSS itself and/or its induction of a “hypercarcinogenic state” on the carcinogenic behaviors of various other factors either intrinsic or extrinsic, including environmental chemicals, are expected to be studied using appropriate in vivo animal models as well as the modifying effects due to various natural and synthetic agents. KBrO₃ can serve as a good model compound, because it is a “genotoxic” carcinogen causing RONOSS during its carcinogenicity, it therefore no longer being used as a food additive. The hepatocarcinogenesis model in rats fed the CDAA diet also provides a good tool, because it causes a “hypercarcinogenic state” closely resembling the human “counterpart” of chronic hepatitis. It is strongly expected that studies on the roles of RONOSS in carcinogenesis using KBrO₃, the CDAA diet and various other tools will provide information useful for the understanding of the carcinogenic mechanisms and in turn the establishment of the practical strategy to control cancers.

References

Epe B (2002). Role of endogenous oxidative DNA damage in carcinogenesis: What can we learn from repair-deficient mice?. *Biopolymers*, 383, 467-75.
Oxygen Species Induced Stress


Sai K, Takagi A, Umemura T, et al (1991). Relation of 8-hydroxydeoxyguanosine formation in rat kidney to lipid peroxidation, glutathione level and relative organ weight after...
Umeda T, Hino O (2002). Molecular aspects of human hepatocarcinogenesis mediated by inflammation: from hypercarcinogenic state to normo- or hypocarcinogenic state. Oncology, 62 Suppl 1, 38-42.