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Meeting Report

Oxy Radicals and Antioxidative Responses in Cancer: 12th Sapporo Cancer Seminar[1]

Life in an aerobic environment provides organisms with an enormous benefit, the high ATP yield of oxidative phosphorylation, but also presents significant dangers. Thus, whereas oxidative phosphorylation results mainly in the four electron reduction of dioxygen to water, a small fraction of the mitochondrial electron flow (3–5%) as well as several other physiological and pathophysiological processes result in only the partial reduction of oxygen. The products of partial reduction are superoxide, hydrogen peroxide, and hydroxyl radical, corresponding to the addition of one, two, and three electrons to dioxygen, respectively. These molecules, along with singlet oxygen, comprise the potentially damaging products classically referred to as ROS[2] or reactive oxygen intermediates. Quite recently NO, originally identified as an endothelium-derived relaxing factor, was also found to be an important oxy radical and reactive oxygen intermediates.

In the body there are many important protective enzymes available to detoxify these ROS, including catalase, glutathione peroxidase, and superoxide dismutase. Small molecular weight antioxidants such as glutathione and ascorbic acid are also important.

In terms of the DNA damage and carcinogenic effects due to ROS, the formation of OH^8Gua is an important factor. Altered gene expression attributable to ROS and the vital role of oxidation-reduction-sensitive transcriptional factors in gene expression are also central issues in this area.

The subject of the 1992 Sapporo Cancer Seminar held on July 15–17, 1992, in Sapporo, Japan, was "Oxy Radicals and Antioxidative Responses in Cancer" organized by N. Taniguchi, S. Nishimura, C. B. Pickett, and O. W. Griffith.

Glutathione and Ascorbic Acid

Glutathione and ascorbic acid are functionally and quantitatively important antioxidants and "old and new compounds" in terms of oxy radical research. The synthesis of glutathione occurs intracellularly and is catalyzed by two enzymes, one of which, γ-glutamylcysteine synthetase, plays a key role in the regulation of glutathione metabolism. In cisplatin-resistant ovarian cancers, high expression of γ-glutamylcysteine synthetase accounts for the high levels of glutathione observed. Inhibition of γ-glutamylcysteine synthetase by buthionine sulfoximine, a specific inhibitor of the enzyme, is expected to cause depletion of GSH and to reverse the cisplatin resistance. Preliminary clinical trials based on this approach are planned or are in progress. A similar elevation of glutathione may occur in the development of resistance to other drugs and to radiation therapy. In addition, other antioxidants may also be important. Recent studies indicate that ascorbate both spares glutathione and provides significant antioxidant protection in glutathione-deficient tissues (a plenary lecture by Dr. A. Meister, Cornell University Medical College, New York, NY).

Dr. T. Kondo (Nagasaki University, Nagasaki, Japan) also reported that in cisplatin-resistant lung cancer cells both γ-glutamylcysteine synthetase and glutathione *S*-transferase activities are elevated. GSH levels, however, decreased when the cells were exposed to cisplatin. Cisplatin is found inside cells as the GSH *S*-conjugate, a species which is transported through GSH conjugate-stimulated ATPase.

Regulation of Glutathione *S*-Transferase and Quinone Reductase Gene

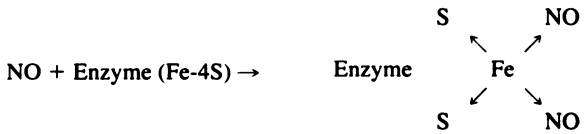
Glutathione *S*-transferase P, originally reported by the late Dr. Kiyomi Sato (Hirosaki University, Hirosaki, Japan), was found to be the best marker for preneoplastic lesions of primary hepatoma in rodents. The transferase gene has been cloned by Dr. M. Muramatsu and coworkers (Saitama Medical College, Saitama, Japan), who demonstrated that the gene contains a strong enhancer (GPE1) 2.5 kilobases upstream from the transcription start site. The GPE1 has a palindrome-like structure which is composed of two TPA-responsive elements and binds at least three proteins including AP-1 (the heterodimer of c-Jun/c-Fos). The gene has also a silencer which binds at least three proteins including SF-B/LAP/LIP.

The glutathione *S*-transferase *Ya* subunit gene and the quinone reductase gene are transcriptionally activated by planar aromatic compounds such as 3-methylcholanthrene and β-naphthoflavone as well as phenolic antioxidants such as *tert*-butylhydroquinone. The ARE has been identified in those genes (Dr. C. Pickett, Merck Frosst Center, Quebec, Quebec, Canada).

Point mutagenesis revealed that the ARE has a core sequence of 5′-GTGACNNNGC-3′. The ARE represents part of a signal transduction pathway which leads to transcriptional activation of some phase II drug-metabolizing enzymes by compounds which undergo oxidation-reduction cycling to form ROS.

Nitric Oxide and NO Synthase

NO is a short-lived and highly reactive oxygen species and is one of the most important messenger molecules in cells. Dr. J. Hibbs (University of Utah School of Medicine, Salt Lake City, UT) originally reported in 1987 that following cytokine exposure the cytotoxicity of activated macrophages toward tumor cells is L-arginine dependent. It was found that L-arginine is the substrate of an enzyme synthesizing inorganic nitrogen oxides and L-citrulline. Experiments involving coculture of activated macrophages with leukemic tumor cells indicated that an inducible NO synthase in the activated macrophage produces NO, which binds to and removes iron from Fe-S iron proteins. Destruction of Fe-S centers causes the inactivation of nonheme iron proteins such as aconitase, mitochondrial respiratory chain complex I and II, and quinone oxidoreductase. The binding of NO to Fe-S proteins has been confirmed by identifying the electron paramagnetic resonance signal due to a dithiodinitroso compound as shown.



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[1] We thank the Senri Life Science Foundation, the Uehara Foundation, the Naito Foundation, and the Banyu Pharmaceutical Co., Ltd., for their partial financial support.
[2] The abbreviations used are: ROS, reactive oxygen species; OH^8Gua, 8-hydroxydeoxyguanosine (8-oxo-7,8-dihydroguanine); GSH, glutathione; ARE, antioxidant-responsive element; Fe-S, nonheme; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; SOD, superoxide dismutase; ADF, adult T-cell leukemia-derived factor; HTLV-1, human T-cell leukemia virus 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide.

Dr. Hibbs also discussed the significance of NO in mammalian systems. Tumor-bearing mice treated with *Bacillus Calmette-Guérin* or cancer patients treated with IL-2 produced large amounts of urinary nitrate (NO_3^-), the degradation product of NO and thus a marker for endogenous NO production by NO synthase. The studies confirmed the presence of a cytokine-inducible, high output L-arginine:NO pathway in humans. He also emphasized the importance of the possible reaction of superoxide anion with NO to form peroxynitrite, a reactive species that can also produce highly toxic hydroxyl radical. Administration of LPS and *Propionibacterium acnes* to rats resulted in the excretion of a large amount of nitrite (NO_2^-) and NO_3^- in urine; this excretion was blocked by treatment with N^{ω}-methyl-L-arginine, a potent inhibitor of NO synthesis (Dr. H. Esumi, National Cancer Center Institute, Tokyo, Japan). NO synthase was purified from livers of rats given LPS and *P. acnes*, using 2′,5′-ADP-agarose affinity chromatography. Both Ca^{2+}/calmodulin-dependent and -independent NO synthases were identified in liver. Peptide mapping analysis and complementary DNA cloning indicated that the two forms are indistinguishable and probably consist of the same protein; the differing sensitivity to Ca^{2+} must therefore be due to factors other than the primary sequence.

Dr. O. Griffith (Medical College of Wisconsin, Milwaukee, WI) reported studies of the hypotensive effects of treatment with cytokines such as TNF, IL-1, and IL-2 in dogs. N^{ω}-Methyl-L-arginine restored blood pressure, indicating that the hypotension caused by these cytokines is mainly or entirely due to the overproduction of NO. In separate studies, arginase was administered to guinea pigs or rats to limit substrate availability for NO synthase in hypotensive states caused by overproduction of NO. Arginase administration also restored blood pressure. These studies indicated that in cytokine-induced hypotension or septic shock, blood pressure can be restored by treatment with NO synthase inhibitors or by limitation of substrate availability.

DNA Damage Due to ROS and Fenton Chemistry

Dr. B. Halliwell (University of California Davis Medical Center, Sacramento, CA) summarized the significance of reactive oxygen species as a "double-edged sword" in biological systems. The superoxide-driven Fenton reaction is very important in the production of hydroxyl radicals.

$$Me^{+n} + O_2^- \rightarrow Me^{+(n-1)} + O_2$$

$$Me^{+(n-1)} + H_2O_2 + H^+ \rightarrow Me^{+n} + OH^{\cdot} + H_2O$$

In the second equation, the reaction of Cu^+ with H_2O_2 is much faster than Fe^{2+} with H_2O_2. Since NO releases iron from Fe-S proteins, it may stimulate Fenton reaction. In addition, as noted NO interacts with superoxide anion radical to produce peroxynitrite.

In health ROS formation is balanced by antioxidant defenses. However, under increased oxidative stress, such as might occur due to poor diet/malabsorption or in various diseases including acquired immunodeficiency syndrome, there can be increased ROS generation and insufficient antioxidant defenses to cope with the ROS. A very early event in such oxidative stress states results in DNA damage characterized by the formation of OH^8Gua. Lipid peroxidation is a later event. Dr. H. Kasai (National Cancer Center Research Institute, Tokyo, Japan) emphasized the importance of OH^8Gua formation in target tissues by ROS-producing carcinogens such as $KBrO_3$ and simfibrate and by choline-deficient diets. The OH^8Gua in DNA in fact induces GC to TA transversions *in vitro* and *in vivo* in the *Escherichia coli* system as reported previously by several workers. The presence of enzymes responsible for repair of the OH^8Gua residue in DNA in mammalian cells has been reported. The formation of OH^8Gua by photosensitization in the presence of riboflavin has also been characterized.

Dr. M. K. Shigenaga (University of California, Berkeley, CA) developed polyclonal and monoclonal antibodies to OH^8Gua to enable noninvasive detection of this species. Studies with model systems, including animals treated with peroxisome proliferaters, depletion of glutathione by BSO, and use of transgenic mice expressing the hepatitis B viral antigen, all support the view that mitogenesis and oxidative DNA damage may play a significant role in the mechanism of carcinogenesis.

Dr. E. Ohtuska and her group (Hokkaido University, Sapporo, Japan) constructed a synthetic c-Ha-*ras* protooncogene containing OH^8Gua in the second position of codon 12 (GCC) and transfected that mutated gene into NIH3T3 cells. The transfection of the gene was found to be associated with significantly increased numbers of transformed foci. The sequence analysis of the c-Ha-*ras* present in the foci indicated that majority of the mutation was G to T transversion as in the case of *E. coli*, but a significant number of mutations was found in the modified site (G to A and G to C) as well as at the adjacent site.

The administration of $KBrO_3$ to rats resulted in an increase of OH^8Gua in kidney and this increase was diminished by treatment with cysteine or vitamin C. Singlet oxygen has been implicated in this process (Dr. Y. Kurokawa, National Institute of Hygienic Sciences, Tokyo, Japan).

Dyes such as methylene blue are known to damage guanine in DNA and RNA and to produce singlet oxygen in the presence of light. Hematoporphyrin D causes a similar effect. The photo-induced formation of OH^8Gua is inhibited by azides, a singlet oxygen quencher. It was shown, however, that the killing of the RNA virus QB by methylene blue plus light did not involve either RNA strand break or the formation of OH^8Gua. The presence of G to T as well as G to C transversions in the above system indicates that there is another guanine adduct in addition to OH^8Gua (Dr. R. A. Floyd, Oklahoma Medical Research Foundation, Oklahoma City, OK). Dr. M. Sekiguchi (Kyushu University, Fukuoka, Japan) has isolated an enzyme, coded by the *mutT* gene, which specifically hydrolyzes OH^8Gua-dGTP to the monophosphate in *E. coli*. A defect of this gene increases the occurrence of AT to CG transversions 100–10,000-fold above the level seen in wild type cells. The enzyme seems to play an important role in the repair of DNA damage caused by ROS.

Dr. S. Kawanishi (Kyoto University, Kyoto, Japan) proposed that the main active species causing DNA damage are copper-oxygen complexes having reactivity similar to that of singlet oxygen and/or hydroxyl radical. In the presence of Cu^{2+}, hydrazine and its derivatives caused site-specific DNA damage. The copper-oxygen complex, rather than the hydroxyl radical itself, derived from the reaction of H_2O_2 with Cu^+ participates in the DNA damage.

Dr. L. A. Loeb (University of Washington, Seattle, WA) reported studies in which single-stranded M13mp2 DNA containing a target gene, *lacZα*, was incubated with an oxygen radical-generating system; mutations were then scored by transfecting the DNA into SOS-induced *E. coli*. He concluded that human neutrophils produce mutagenic ROS, which induce tandem double CC-TT mutations in DNA. The CC to TT mutation may be a diagnostic marker for DNA damage caused by ROS.

Mutagenesis and Carcinogenesis Due to ROS

Dr. Y. Konishi (Nara Medical University, Nara, Japan) observed the effect of antioxidant drugs on lipid peroxidation in γ-glutamyl transpeptidase-positive foci in livers of rats fed a choline-deficient, L-amino acid defined diet. An ascorbic acid derivative, CV-3611 (2-*O*-octadecylascorbic acid), *N*,*N*′-diphenyl-*p*-phenylenediamine, buty-

lated hydroxytoluene, and various other antioxidants were all effective in preventing lipid peroxidation and transpeptidase-positive foci formation. His group (Dr. A. Denda) also reported that increased oxidative stress such as that caused by depletion of glutathione, induction of cytochrome P-450, or menadione treatments resulted in an increase of OH^8Gua and hepatocyte necrosis.

Antioxidative enzyme activity is decreased in human gastric, colon, and liver cancers (Dr. S. Iinuma, Kyoto Prefectural University of Medicine, Kyoto, Japan). The involvement of ROS in the hepatocarcinogenesis was also suggested by studies with LEC rats, a mutant strain with high tissue copper levels similar to those seen in Wilson's disease. Such rats develop spontaneous hepatitis and hepatomas (Dr. H. Sone, National Cancer Institute, Tokyo, Japan). Coenzyme Q_{10} inhibited the formation of malondialdehyde and decreased liver transaminase activities in the LEC rats. Dr. M. Sagai (National Institute of Environmental Studies, Tsukuba, Japan) suggested that chronic exposure to O_3, NO_2 or NO_3/H_2SO_4 as pollutants has a synergistic effect on their activity as tumor promoters in lung cancer.

Dr. H. Maeda (Kumamoto University, Kumamoto, Japan) reported that superoxide anion is generated by highly purified cytochrome P-450 reductase and NADPH acting on 11 different heterocyclic amines, including aminodimethylimidazoquinoline and aminomethylimidazoquinoline. He found a positive correlation between the amount of DMPO-OOH generated as judged by electron paramagnetic resonance and mutagenicity as judged by the Ames test. He also reported a significant correlation between the antitumor-promoting effect of green leafy vegetables such as green perilla and various beans in an Epstein-Barr virus/B-lymphocyte phorbol ester system and the lipid radical-scavenging effect of the foods. Dr. M. Hosokawa (Hokkaido University, Sapporo, Japan) reported on the role of ROS in tumor progression by using a spontaneously regressive tumor (QR 32) which is derived from mouse fibrosarcoma. When exposed to host effector cells *in vitro* or *in vivo*, the cells are easily converted to more malignant phenotypes characterized by production of prostaglandin E_2, rapid growth, and metastatic properties. Effector cells generate ROS which causes conversion.

New Insights of Cu,Zn-Superoxide Dismutase and Mn-Superoxide Dismutase

Drs. H. F. Deutsch and M. A. Evenson (University of Wisconsin, Madison, WI) used high voltage capillary electrophoresis to characterize the heterogeneity of various Cu-, Zn-, and Mn-superoxide dismutases including human recombinant enzyme. Enzymes were purified and crystallized from human and bovine tissues. High voltage capillary electrophoresis can detect 0.4-ng or 10-fmol quantities and is a powerful tool for the detection of minor contaminations in SOD preparations.

Dr. M. Ishikawa (Asahikawa Medical School, Asahikawa, Japan) reported that Mn-SOD is released into the blood stream in approximately 60% of patients with epithelial ovarian cancer. Serum Mn-SOD is a good marker for monitoring and diagnosing ovarian cancer, a tumor difficult to diagnose at early stages. Histological studies of Mn-SOD in the ovarian cancer were also reported (Dr. T. Nishida, Kurume University, Kurume, Japan). The mechanism by which tumor tissues produce cytokines such as IL-1 and TNF, which stimulate the production of Mn-SOD in vascular endothelial cells was also discussed (Dr. T. Nakata, Osaka University Medical School, Osaka, Japan). Mn-SOD is also elevated in patients with neuroblastoma (Drs. N. Kawamura and K. Suzuki, Osaka University, Osaka, Japan).

Dr. J. McCord (University of Colorado Health Science Center, Denver, CO) proposed that the dose-response curve is very important in the design and use of SOD in the treatment of ischemic heart disease. The bell-shaped dose-response curve indicates that too much SOD may interfere with important superoxide-requiring reactions and be counterproductive. He also proposed that iron liberated from ferritin by superoxide anion is a likely candidate for the initiator of lipid peroxidation. Stored iron levels in human serum may be a critical factor and marker in cardioischemic diseases. He emphasized the importance of zinc finger motifs, which bind to enhancer regions of various genes that are activated by ROS.

Dr. Y. Niitsu (Sapporo Medical College, Sapporo, Japan) reported previously that ROS are implicated in tumor cell killing by TNF. He transfected nonsecretory sense and antisense TNF genes into TNF-sensitive mouse fibroblastic cells (L-M cells) to examine whether TNF exerts its protective function directly in the cells or in an autocrine manner. He found that TNF, hyperthermia, and anticancer drugs such as Adriamycin may produce ROS that cause cell lysis. Against these stresses, endogenous TNF may induce Mn-SOD production to protect against cell lysis.

Dr. N. Taniguchi (Osaka University Medical School, Osaka, Japan) reported previously that Cu,Zn-SOD undergoes a glycation reaction (the Maillard reaction) and inactivation under hyperglycemic conditions and aging. In patients with insulin-independent diabetes, a large amount of the glycated form of Cu,Zn-SOD was found. His group (Dr. T. Ookawara *et al.*) identified a site-specific cleavage at Pro 62-His 63 as well as random fragmentation due to the glycation. This fragmentation is due to the production of hydroxyl radicals by Fenton chemistry. The induction mechanism of the Mn-SOD gene by TPA, TNF, and LPS in various TNF-resistant cell lines was also reported (Dr. J. Fujii, Osaka University, Osaka). It was proposed that protein kinase C-dependent and -independent signal transduction pathways exist to induce the Mn-SOD gene. Dr. Y. Mogi (Sapporo Medical College, Sapporo) reported that SOD has marked inhibitory effects on the pulmonary metastasis of murine tumor cells; therapeutic use of SOD was suggested.

Altered Gene Expression by ROS and Transcriptional Factors

In some transplantable hepatomas in culture and in tumor-bearing rats a marked decrease in catalase activity is observed. In order to investigate the mechanism at the gene level, Dr. H. Endo (Tottori University, Yonago, Japan) determined that the reduction of catalase activity is due to decreased gene transcription. An important *cis*-acting element and a silencer element (5′-TGGGGGGAG-3′) were identified in the 5′ flanking region of the catalase gene. The same core sequence was also found in various liver enzyme genes such as those for aldolase B, ornithine transcarbamylase, and albumin. All were reported to be down-regulated in hepatoma. A M_r 35,000 nuclear factor was found in ascites hepatoma AH-66 cells and this fact may be involved in the negative regulation of catalase gene expression.

NF-κB is an inducible transcription factor that is activated when cells are exposed to TNF, IL-1, viruses, double-stranded RNA, LPS, UV light, and γ-rays. In a human T-cell line, H_2O_2 activates NF-κB and the activation was blocked by catalase, *N*-acetyl-L-cysteine, and various non-sulfur iron/copper chelators. Tax protein from HTLV-1, the X protein from hepatoma B virus, and truncated forms of the middle surface antigen from hepatoma B virus, referred to as MHBs, all activate NF-κB as well as a series of other host transcription factors via increased levels of ROS (Dr. P. A. Baeuerle, Ludwig-Maximilil-ians University, Munich, Germany).

Dr. K. Nose (Showa University, Tokyo, Japan) reported that ROS such as superoxide anion and H_2O_2 are produced in response to platelet-derived growth factor and TPA in cultured fibroblasts and epithelial cells. The ROS modulate positively or negatively DNA synthesis depending on cell cycle or cell types. His group (Dr. M. Shibanuma, University of Tokyo, Tokyo, Japan) also proposed that

H_2O_2 is one of the second messengers for signal transduction of TGF-β_1 in late G_1 phase of the cell cycle.

Treatment of bacterial cells with low doses of H_2O_2 results in the expression of many proteins and allows development of resistance to killing by higher dose of H_2O_2. The expression of nine of the inducible proteins is controlled by the regulater OxyR, which is both a negative regulator of its own expression and a positive regulator of the inducible proteins. The OxyR protein binds to the promoters of oxyR-regulated genes. OxyR controls also the expression of a 107-nucleotide, noncoding RNA, and acts to positively and negatively regulate genes in response to oxidative stress (Dr. G. Storz, National Institute of Child Health and Human Development, Bethesda, MD).

Dr. J. Yodoi (Kyoto University, Kyoto, Japan) reported that HTLV-1-transformed T-cells overexpress IL-2 receptor α chain and ADF. ADF is homologous to human thioredoxin, an endogenous thiol-reducing protein, and is expressed at high levels in cellular transformations following infection by HTLV-1, Epstein-Barr virus, and human papilloma virus. Oxidation-reduction regulation via ADF/thioredoxin may play an important role in the activation of NF-κB. Adriamycin also protects the cells from TNF as well as H_2O_2 cytotoxicity.

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