Review Article

The origin and future of oxidative stress pathology: From the recognition of carcinogenesis as an iron addiction with ferroptosis-resistance to non-thermal plasma therapy

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Helmut Sies established the concept of oxidative stress in 1985. However, it took some time to introduce this concept into pathology, where investigators count on formalin-fixed paraffin-embedded tissue sections. I sought out antigens for this purpose based on an oxidative stress-induced rat renal carcinogenesis model, which revealed that 8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins are ideal. These two monoclonal antibodies successfully revealed the involvement of oxidative stress in numerous human diseases, including carcinogenesis and atherosclerosis. Shigeru Okada established the aforementioned ferric nitritotriacetate (Fe-NTA)-induced rat renal carcinogenesis model, which thus far has answered many questions regarding the presence of target genes in oxidative stress-induced carcinogenesis and the sites that are susceptible to oxidative stress in the genome. Particularly, the similarity of genomic alterations between Fe-NTA-induced renal cancer and human cancers suggests that excess iron plays a role also in human carcinogenesis. Furthermore, excess iron is a major pathology in asbestos-induced mesothelioma, including chrysotile. Despite an analogy to asbestos, multi-wall carbon nanotubes were distinct in that diameter is another responsible factor for mesothelial carcinogenesis. Recently, non-thermal plasma emerged as a candidate for medical intervention for wounds and cancers via manipulating oxidative stress. Counteracting excess iron is a promising preventive strategy for major diseases.

Key words: carcinogenesis, free radicals, iron, monoclonal antibody, oxidative stress

INTRODUCTION

We cannot live without oxygen, even for 5 min. However, it is difficult to recognize what oxygen is actually doing in our body after being taken up from the ambient air. The first life on earth appeared approximately 3.7 billion years ago, when the oxygen concentration at the earth’s surface was very low. Thus, the first life is thought to have been an anaerobe-mimic. Two and a half billion years ago, the oxygen concentration in the air started to increase as solubilized ferrous iron (Fe[II]) in the sea was piled up at the sea bed as Fe(OH)₃ and finally as Fe₂O₃, an iron ore, leading to low soluble iron environment in the sea. These events appear to have been mediated by algae. Since this era, iron and oxygen have presented a high affinity for each other on earth. Increased molecular oxygen in the air should have been toxic to the preexisting species. Under this original oxidative stress, they were forced to make decisions leading to three possible outcomes: to become extinct, to retreat to anaerobic environments or to generate novel systems against oxidative stress. Now, we instantly understand which decision was the optimal for evolution.

Aerobes and higher species make use of the distinct characteristics of molecular oxygen (O₂) so that it can work as an electron acceptor as many as four times independently during the reduction process to H₂O. Thus, O₂ is a means of electron flow (Fig. 1). As we can see, almost all moving objects, living organisms or machines, use electron flow systems, such as mitochondria, battery cells and motors. Steam locomotives that use coal and do not use electron systems show very low energy efficiency as well as huge CO₂ emission.

Free radicals are defined as any chemical species with one or more unpaired electron(s), and free radical reactions have been recognized since the 1930s, when the reactions were practically used to generate synthetic rubber and plastic. Atomic bombs generated in the US emit radiation, heat and blast, which were used to attack Nagasaki and Hiroshima in Japan during World War II. Detailed epidemiological data on the attributable consequences offer strong evidence that free radical reactions by radiation causes cancers, such as leukemia in the early years and various solid tumors in the late years.
These lines of research have been conducted intensively in Japan since 1945 up to the present day. In 1956, Harman, for the first time, proposed that one factor in aging may be related to deleterious side attacks of free radicals. Additionally, it was when Fridovich and McCord reported superoxide dismutase in 1969 as the first enzyme to use free radicals as a substrate that researchers started to realize that free radicals always exist in our body. Of note, superoxide dismutase catalyzes the reaction of altering two superoxide molecules into oxygen and hydrogen peroxide.

Oxidative stress

In 1985, Helmut Sies coined the term “oxidative stress” when he edited a book of the same title. This scientific phrase is now so famous that there are 145 957 hits in the PubMed database with an ever increasing trend and approximately 2 050 000 hits in Google scholar (January 17, 2016). This book covers the framework of the current oxidative stress biology, starting from radiation-induced DNA damage, oxygen toxicity augmented by various chemicals, mitochondria, antioxidant vitamins and its equivalents in foods, chemical reactions in lipid peroxidation, inflammation, neurodegenerative diseases and finally cancer. This book indeed boosted the research of oxidative stress and free radicals in biology and medicine, and its publication year is miraculously identical to my graduation year of medical school.

On the way to four serial one-electron reductions to H₂O, oxygen goes through superoxide, hydrogen peroxide and a hydroxyl radical (Fig. 1). The former two are produced via enzymatic reactions whereas the last one is generated through a chemical reaction in our body. Radiation can directly induce hydroxyl radicals from water through its high energy though this occurs in special occasions such as power plant accidents, cancer therapy and space missions. Singlet oxygen is also special in that one of the major pathways of its generation is through ultraviolet or light with natural/synthetic pigments, such as porphyrin and methylene blue. Thus, we may conclude that life on earth has been avoiding radiation and ultraviolet light for it to prosper.

Among the three intermediates between oxygen and water (Fig. 1), the reactivities of superoxide and hydrogen peroxide per se are not high but work for reversible reduction/oxidation (redox) reactions except for further reactions with a large amount of nitric oxide or ferrous iron, respectively, as will be described later. However, the hydroxyl radical is the most reactive species in the biological system, causing covalent modifications to many biological molecules (DNA, RNA, protein, sugar, etc.) such as scission, additions and cross-links. This chemical reaction of hydroxyl radical generation is catalyzed by catalase, peroxidases and peroxiredoxin prevent the generation of dangerous hydroxyl radicals. Biological effects of oxidative stress depend on its intensity, duration and cell-type, ranging between cell death and proliferation. Refer to text for details. NO, nitric oxide.

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by ferrous iron (which now can be visualized with a RhoNox-1 probe\cite{26,27} and is called the Fenton reaction.\cite{28} Accordingly, it is not surprising that we have many different types of enzymes to decompose hydrogen peroxide directly to water by bypassing the Fenton reaction. These enzymes include catalase,\cite{29} peroxidases,\cite{30} peroxiredoxins\cite{14,31} and sulfiredoxin\cite{32} (Fig. 1).

Starting from the late 1980s, it was gradually recognized and established that some of the reactive species, such as superoxide, hydrogen peroxide and nitric oxide, work as signaling molecules in the cell. The important point was reversibility of the reaction, which was attained on the sulfhydryl group of cysteine residues (Fig. 1). Indeed, sulfhydryl groups can be oxidized to sulfenic, sulfenic,\cite{33,34} sulfonic,\cite{35,36} and sulfonic acids, the former two of which could be reduced to sulfhydryls. Glutathione and thioredoxin systems have been implicated in such reactions,\cite{37} but this was further extended to other peptides and proteins, especially transcription factors. Particularly, the Nrf2 transcription factor in combination with the Keap1 oxidative stress sensor,\cite{38,39} first reported in 1999, has gained universal scientific attention as a system that immediately responds to oxidative stress by transcribing genes to counteract oxidative stress.\cite{40,41} The emergence of the concept of oxidative stress as a cellular signaling process prominently increased the studies on oxidative stress.

We currently understand that the biological effects of oxidative stress are dose-dependent. Prooxidants are represented by the generation of reactive species whereas antioxidants generally act in three distinct ways: preventing initiation, blocking the propagation of free radical reactions and repairing the covalently modified molecules by free radical reactions. In principle, high oxidative stress causes cellular necrosis; moderate oxidative stress causes apoptosis and a low level of oxidative stress causes cellular proliferation\cite{21,42} (Fig. 1). In contrast, the normal steady state is mildly reductive, as represented by pH 7.4, and cells accomplish their own function according to their differentiation.

**Visualization of oxidative stress**

Formalin-fixed paraffin-embedded (FFPE) tissue blocks/sections are precious in that they can be preserved at room temperature practically forever, present morphological pathogenesis information at a single-cell level and still retain a variety of bioinformation, such as genome, mRNA and proteins if properly fixed.\cite{44} FFPE specimens are currently used for next-generation sequencing. In the late 1980s, my mentor Osamu Midorikawa, a professor of pathology, often told us during the regular department seminar that free radicals/oxidative stress are not interesting because we cannot see them and even questioned the existence of free radicals. In those days, the only available method was electron spin resonance with spin trapping. This method is still used currently\cite{45,46} but is a physical method, revealing little about morphology. During my days as a graduate student, I was always dreaming of novel methods to visualize oxidative stress in FFPE.

In the 1980s, the Department of Pathology, Faculty of Medicine, Kyoto University was attempting to produce various animal models, simulating human diseases under Osamu Midorikawa. Among them, Shigeru Okada, who was interested in iron metabolism stimulated by his mentor, Sachimaru Seno, was trying to reproduce the results of Michiyasu Awai, which were inspired by a previous biochemical study,\cite{47} in which ferric nitritetriacetate (Fe-NTA) injected intraperitoneally can be deposited in parenchymal cells, such as hepatocytes and \( \beta \)-cells in pancreatic Langerhans islet,\cite{48} to evaluate the iron excretion from the biliary system. Before that report, any iron compounds used were deposited in the reticuloendothelial system, including Kupffer cells and macrophages but did not go to the parenchymal cells. Thus, the report by Awai was surprising. After reproducing those experiments using rats, Shigeru Okada unintentionally let the animals survive, and the animal facility technician continued to feed them regularly. One day after 1 year had passed, the technician noticed that several of the animals were dying with enlarged abdomens and reported this observation to Shigeru Okada. With an autopsy, he found that many of the dying animals had large renal tumors that often metastasized to the lung and invaded the peritoneal cavity.\cite{49} Thereafter, the Fe-NTA-induced renal carcinogenesis model was firmly established both in rats\cite{50} and mice.\cite{51} Thus, when I joined as a PhD student, the atmosphere of the department was cheerful. Shuji Hamazaki noticed the renal oxidative damage as early as 20 min after Fe-NTA administration,\cite{52,53} Thus, we hypothesized that Fe-NTA works as a catalyst for the Fenton reaction in vivo. Then, the study on vitamin E (\( \alpha \)-tocopherol) pretreatment confirmed the hypothesis.\cite{54}

In 1987, Alfonso Pompella, for the first time, used cold Schiff reagent to visualize lipid peroxidation in pathology specimens.\cite{55} Most of the final products of lipid peroxidation are aldehydes,\cite{56} and cold Schiff reagents reacted with aldehydes to confer a pink color. I applied this method to the frozen renal sections obtained from a kidney 30 min after intraperitoneal Fe-NTA administration and found that aldehydes are generated immediately after glomerular filtration, inducing oxidative damage to renal proximal tubules.\cite{57–59} The only problem was that we had to use frozen sections, not FFPE specimens. At that time, many researchers were trying to generate monoclonal antibodies, which have merits over polyclonal antibodies as permanent properties. Thus, I also decided to make monoclonal antibodies to detect oxidative stress in FFPE specimens. I wondered what the best antigens are for this purpose. However, it was not easy to obtain the answer immediately. The antigens had to be maintained after formalin fixation and paraffin embedding, so lipids were not appropriate. Additionally, the antigens should present a high signal/background ratio under oxidative stress compared to...
normal controls, which obviously required certain comprehensive analyses (Fig. 2).

I had a chance to go to the US as a postdoctoral fellow in the Center for Devices and Radiological Health, Food and Drug Administration under the guidance of Jose-Luis Sagripanti during 1990–1992. Studying in foreign countries provides a special opportunity to meet many people with different expertise, which may be contrasted by a short stay for a scientific meeting. I met Koji Uchida, Earl Stadtman, Jim Strickland, Miral Dizdaroglu and Denis C Lehotay, with whom we collaborated even after I came back to Japan, and these efforts finally resulted in what I needed for oxidative stress pathology. Koji Uchida and Earl Stadtman taught me chemical reactions of aldehydes derived from lipid peroxidation, Jim Strickland gave me lessons on how to use a reference manager software, and Miral Dizdaroglu and Denis C Lehotay presented us with mass spectrometric analyses of oxidative DNA modifications and aldehydes, respectively. Here, the important point was that I used the aforementioned Fe-NTA-induced renal carcinogenesis model for the selection of the two antigens. One of the antigens for the monoclonal antibody was 8-hydroxy-2′-deoxyguanosine, which increased 6.2-fold and increased the most 3 h after Fe-NTA administration out of the 10 oxidatively modified DNA bases. The other antigen was 4-hydroxy-2-nonenal (HNE)-modified protein(s). HNE increased 27.3-fold and increased the most 3 h after Fe-NTA administration out of

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**Figure 2** Roadmap to establish monoclonal antibodies for recognizing oxidative stress in formalin-fixed paraffin-embedded tissue specimens, which was accomplished in the 1990’s. Refer to text for details.
the 26 aldehydes and their analogues. Aldehydes, including HNE, are lipids and are not retained in the FFPE specimens. However, HNE is so reactive that it further reacts with cysteine, histidine or lysine residues in proteins to generate Michael products, which are retained in the FFPE specimens. In this way, we produced two monoclonal antibodies (clone N45.166 and clone HNEJ-267), which played a role in establishing the involvement of oxidative stress in a variety of pathologic situations using FFPE specimens (Fig. 2). Typical examples include type-2 diabetes mellitus, UV-induced epidermal damage and arsenic-induced skin injury. These monoclonal antibodies are commercially available and have been monopolized in hundreds of studies.

**Oxidative stress and cancer**

Many pathologic conditions can induce oxidative stress in tissues and cells. These conditions, summarized in Table 1, are all associated with a risk for cancer except for ischemia-reperfusion injury, where acute effects are presumably so predominant that the incubation period for carcinogenesis would not be sufficient. Fig. 3 presents the current classification of carcinogenic agents. I believe that these items are of the tip of the iceberg and that the huge bottom portion of the iceberg consists of endogenous oxidative stress derived from the use of oxygen and iron. Iron is the most abundant heavy metal, and adult human males contain approximately 4 grams, 60% of which are present as the heme of hemoglobin in red blood cells. No life on earth can live without iron thus far. Because iron has been so important and precious, we do not have any active metabolic pathways to excrete iron outside of our body once it is absorbed via the duodenal mucosa via DMT1 or if it is injected parenterally. Iron excretion is normally attained only through hemorrhage or cellular loss from the skin or mucosa, which is approximately 1 mg per day whereas 1 mg of iron is absorbed from diets through duodenal epithelial cells. Thus, iron metabolism is a semiclosed system. In contrast, iron circulation in the blood is very fast, considering that the lifetime of red blood cells in humans is approximately 120 days, so approximately 0.8% is degraded every day in the spleen or other reticuloendothelial system. Iron is taken out of red blood cells by splenic macrophages and is sent to the bone marrow via serum transferrin, which adds up to approximately 19.2 mg per day. Many pathologic situations cause iron excess in parenchymal cells, including genetic alterations in the iron sensor system (hemochromatosis), repeated hemorrhage in a closed space (ovarian endometriosis), inflammation (chronic viral hepatitis) and foreign body intake (asbestos exposure). Each condition is associated with specific carcinogenesis (Table 2). In this sense, excess iron is the most common and also underscored cause of oxidative stress and carcinogenesis.

Currently, transgenic and knockout techniques for mice and rats have become popular and are often used to demonstrate the function of a gene or sometimes several genes simultaneously. However, we humans are basically wild-type animals except for rare cases of familial cancer syndromes, such as Li-Fraumeni syndrome. In the 1990s, we noticed the high reproducibility of Fe-NTA-induced renal carcinogenesis, especially in wild-type rats, where approximately 90% of male Wistar, Fischer-344 and Brown-Norway strains develop renal cell carcinoma, with half of them metastasizing to the lung. Such a strongly reproducible carcinogenesis model of a highly malignant solid tumor in wild-type animals has never been reported to our knowledge. In addition, an acute phase with Fenton reaction-induced renal tubular damage followed by renal tubular carcinogenesis is highly reproducible. Regarding mice, we obtained a much lower incidence of carcinogenesis: 60% in A/J mice and 10% or less for C57BL/6 mice (unpublished data). There is a definite strain difference in the case of mice, which requires further investigation.

**Target genes of oxidative stress-induced cancer**

Hydroxyl radicals are a major chemical species responsible for Fe-NTA-induced renal carcinogenesis and are the most reactive species in the biological system. Therefore, they react with many types of biomolecules, leading to the generation of aldehydes and oxidatively modified DNA bases as mentioned earlier, in addition to DNA/protein strand breaks and cross-links. This is apparently a complex reaction that is difficult to be clarified completely. However, it is true that repeated Fenton reactions in vivo produce cancer as exemplified by Fe-NTA-induced renal carcinogenesis. My question in the 1990s was whether there are any target genes in this oxidative stress-induced carcinogenesis.

Because there was no whole genome data of rats available in the 1990s, we first used a microsatellite analysis strategy to determine the common target genes. We crossed two distant strains of rats (Fischer-344 and Brown-Norway) to obtain F1 rats, which we used for carcinogenesis experiments with Fe-NTA. After thousands of PCR reactions, we reached the
conclusion that the \( p15/p16 \) tumor suppressor genes are a major target genes. Homozygous deletion and hemizygous deletion accompanied by methylation of the promoter region were observed in one third each of the cases of Fe-NTA-induced renal cell carcinoma. The drawback of this strategy was that we could not find the amplified oncogenes.

Coming into the new century, information on the whole genome of rats has become available, and we could use array-based comparative genome hybridization (CGH). We reevaluated the genomic alteration of the Fe-NTA-induced renal cell carcinoma with this method. We confirmed our previous findings, and there were three new findings: first, we found a common target oncogene which was amplified such as \( c\text{-}Met \) (hepatocyte growth factor receptor) and \( Ptprz1 \), secondly, genomic alterations in terms of genomic deletion and amplification were immense at the chromosomal level, which was in contrast with other animal cancer models except for massively genetically engineered mice, and lastly, the genomic alterations were most similar to human renal cell carcinoma, when compared with Oxford grid information with the second most similar being human malignant mesothelioma. I think that our results present many implications. These results suggest that iron excess and the following oxidative stress may also be responsible for human cancers at least partially, especially for the deletion of \( p15/p16 \) tumor suppressor genes (Fig. 4). This story continues to the pathogenesis of asbestos-induced malignant mesothelioma in the subsequent section.

### Oxygenomics

Our current conclusion is that there are target genes, such as \( p15/p16 \), in oxidative stress-induced carcinogenesis as described above. Then, the next question would be whether there are any fragile sites in the genome against oxidative stress. There are many types of DNA damage, including single and double strand breaks, base modifications, abasic sites and cross-links. Most likely, DNA double strand breaks are most closely associated with the amplification and deletion of genes. However, based on our previous data on the close association of 8-OHdG and strand breaks in plasmid DNA and the limitation of current methodology, we focused on the genomic sites that include 8-OHdG. Namely, we performed immunoprecipitation analysis using a monoclonal antibody against 8-OHdG and the binding of 8-OHdG in DNA fragments and N45.1 was strong enough to allow for immunoprecipitation.

In the earlier data, we used the mouse kidney after Fe-NTA administration and sequenced each fragment after cloning, and PCR reactions were also used in combination to evaluate the target genomic loci. We found that the non-gene portion of the genome accumulates more 8-OHdG and furthermore, exposure to oxidative stress or knockout of \( Ogg1 \) (repair enzyme of 8-OHdG) accumulated more 8-OHdG. We also observed that the \( p15/p16 \) loci are relatively more susceptible. I believe that this first observation opened up a novel research area called oxygenomics, which studies fragile genomic loci against oxidative stress (Fig. 4). This phenomenon is presumably associated with chromosome territory, the transcription status of...
the loci and the chemical species involved, which would be different among cell types and pathologic status.

Recently, we have applied this technique with array-based CGH to the genome of the rat kidney, which demonstrated another principle in which 8-OHdG accumulates in the lamina-associated domain, supporting the nuclear membrane structure. Free radicals are abundant in the cytoplasm, so the results confirm the bodyguard hypothesis where the genome near the nuclear membrane protects the rest of the genome deep inside the nucleus. I believe that susceptibility to oxidative stress and the following selective process for proliferation is associated with oxidative stress-induced carcinogenesis.

Ferroptosis

For the past few decades, two types of cell death processes, namely necrosis and apoptosis, have been discussed; necrosis is unintended and passive cell death whereas apoptosis is programmed and strictly regulated cell death. However, recently, this long-standing paradigm in the field of cell death is going to be dramatically changed as new forms of iron-dependent non-apoptotic cell death are emerging. Ferroptosis, a new concept proposed in 2012, is defined as a non-apoptotic programmed cell death that can be inhibited by Fenton reaction inhibitory iron chelators such as desferal. Ferroptosis was first reported to be induced by a set of small molecules in engineered human fibroblasts overexpressing oncogenic H-Ras.

At first, erastin was used to cause this special type of programmed cell death, and lipid peroxidation-derived signals toward cell death were recognized as a hallmark of

Figure 4 Massive chromosomal alterations in Fenton reaction-induced renal cell carcinoma in wild-type rats and the concept of oxygenomics. Refer to text for details. CGH, comparative genome hybridization.
ferroptosis. \textsuperscript{100} Glutathione peroxidase 4 (GPX4) is distinct from the other GPXs in that it directly regulates membrane-associated lipid peroxidation, \textsuperscript{101} and it emerged as a key regulator of ferroptotic cellular death. \textsuperscript{102} The phenotype of its knock-out is infertility due to the low sperm motility. \textsuperscript{103} Further, it was recently reported that the inactivation of GPX4 triggers acute renal tubular cell death. \textsuperscript{104}

These data suggest a central role of membrane-associated iron in ferroptosis. Therefore, the pathogenesis of iron excess-induced carcinogenesis led to one important concept, resistance to ferroptosis, in addition to iron addiction (Fig. 5). \textsuperscript{105-108} Alternatively, cancer cells must resist iron-induced oxidative stress through genetic alteration, require more iron for proliferation and are under persistent oxidative stress. \textsuperscript{105-108}

**Elucidation of asbestos-induced mesothelioma**

Humans have been and continue to be keen on novel products, whether natural or manufactured, that increase the quality of our lives, to the extent that human health risk caused by that novel product is sometimes overlooked due to ignorance or the absence of an appropriate evaluation system. Asbestos is one of those natural products.

Asbestos is a general name for natural fibrous silicate. \textsuperscript{109} Because it is a stone, asbestos is resistant to heat, acids and friction. In addition, asbestos presents versatile textures to be mixed with textile and cement or to be sprayed on any surface. Moreover, there was a marked economic merit with mining. Therefore, a significant amount of asbestos was used all over the world in the last century. However, a rare type of cancer called malignant mesothelioma was recognized by epidemiologists in the 1960s, and the International Agency for Research on Cancer (IARC) declared that asbestos is a definite human carcinogen (Group 1) in 1987 due to accumulated evidence. \textsuperscript{110} Notwithstanding, many countries disregarded the alert of the IARC and continued to use asbestos. Especially, asbestos companies tried to convince scientists and regulators that chrysotile (white asbestos) is much safer than crocidolite (blue asbestos) and amosite (brown asbestos) when handled properly. Canada continued to mine and export chrysotile until the autumn of 2012. \textsuperscript{111} During these situations, “Kubota shock” occurred in Japan in June of 2005. \textsuperscript{112} After this event, our group began to study asbestos-induced mesothelial carcinogenesis. Mesothelial cells, the target of this carcinogenesis, are extremely unique cells in many aspects. \textsuperscript{113,114} In 2006, finally all asbestos was legally banned in Japan and a law was enacted to financially support all of the current malignant mesothelioma patients.

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**Figure 5** General rule on iron metabolism in parenchymal cells in various pathologic conditions. This is based on the fact that iron has been essential and precious for every species during evolution, which eventually leads to an ultimate choice issue in higher organisms with long lifetime. Refer to text for details.

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patients and the families of already deceased patients. Notwith-
standing, the peak year of malignant mesothelioma in Japan is
expected in 2025, and more than 100,000 new patients are ex-
pected in the coming 40 years.115 The situation is similar for
other countries.116 Furthermore, asbestos in the air may in-
crease due to the destruction of old buildings and natural dis-
asters such as hurricanes, tornadoes, earthquakes and tsunamis
in the near future (http://www.asbestos.com/asbestos/natural-
disasters/).

We started with a simple model of intraperitoneal asbestos
administration (10 mg) to rats to expose mesothelial cells di-
rectly to three major commercially used asbestos (chrysotile,
crocidolite and amosite). We also tried intrapleural or
intrapulmonary injections, but we found that intraperitoneal in-
jection was the most sensitive.117 We believe that this finding
is associated with the abundance of adipocytes in the peritoneal
cavity in rats. Adipocytes can promote inflammation through the
production of cytokines such as MCP-1, which can induce a vi-
cious cycle of chronic inflammation by stimulating macro-
phages.118 Other mesenchymal cells may be associated as
well. The data with intraperitoneal injection was striking. Unex-
pectedly, chrysotile was the earliest to induce malignant meso-
thelioma, followed by crocidolite and amosite.117 Eventually,
almost all of the rats developed malignant mesothelioma, and
repeated administration of NTA (Fenton reaction-promoting iron
chelator) promoted mesothelial carcinogenesis in all of the
cases, suggesting the involvement of excess iron in this
carcinogenesis.

Then, we analyzed the genomic alteration of asbestos-
induced malignant mesothelioma. Homozygous deletion of
p15/p16 tumor suppressor genes was observed in most cases
with massive amplification/deletion of the genome,117 which is
almost identical to human counterparts.119,120 This finding also
confirms our hypothesis that asbestos-induced mesothelial car-
cinogenesis is iron-dependent (Fig. 5), based on the similarities
to the target genes in Fe-NTA-induced renal carcinogenesis.87
Whereas this model is skipping the respiratory system, we be-
lieve that this model is also useful to evaluate novel fibrous
nanomaterials for carcinogenicity.

Then, we focused on why chrysotile (white asbestos), which
does not contain iron itself, induces iron-dependent carcinogen-
esis. With time-lapse microscopy, we observed that both mac-
rophages (RAW) and mesothelial cells (MeT5A) are
phagocytic but the following response was quite distinct: macro-
phages die but mesothelial cells do not.121 We comprehen-
sively identified proteins that are adsorbed on three different
types of asbestos fibers.122,123 Common proteins we thought
important were histones, actin, tubulin and hemoglobin. Here,
hemolytic activity and the following adsorption of hemoglobin
by chrysotile appear to be important. With this reaction, chrys-
otile may be armed with iron because hemoglobin is rich in iron
by retaining 60% of all of the body’s iron. In the case of

![current understanding of molecular mechanisms for asbestos-induced mesothelial carcinogenesis](#)

**Figure 6**  Current understanding of molecular mechanisms for asbestos-induced mesothelial carcinogenesis. Annexin A2 plays a role in the uptake of asbestos fibers by mesothelial cells. Refer to text for details.

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crocidolite and amosite, their surfaces work as a catalyst for the Fenton reaction when the fiber interacts with chromatin that contains genomic DNA whereas the hemoglobin coating works as a catalyst in the case of chrysotile. Affinity to DNA was the highest on the surface of chrysotile among the three asbestos. Current understanding of asbestos-induced mesothelial carcinogenesis is summarized in Fig. 6. Based on these results, it is natural to think of iron reduction as a preventive strategy for the people already exposed to asbestos for malignant mesothelioma. With animal experiments, we succeeded in significantly changing the fraction of malignant mesothelioma histology to one with a better prognosis (epithelioid subtype in comparison to sarcomatoid subtype).

In malignant mesothelioma, the histology of the transitional form between epithelioid and sarcomatoid subtypes exists as a biphasic subtype and is included in the official classification. We used an expression microarray to identify genes that are responsible for this transition. The top ranked gene was connective tissue growth factor (CTGF; also as CCN2) and a much higher expression in the sarcomatoid subtype compared to the epithelioid subtype of malignant mesothelioma was observed in rats. However, in contrast to our expectation, CTGF was highly expressed in the epithelioid subtype as well in comparison to non-tumorous mesothelial cells. CTGF plays a role in the maintenance of tumor phenotypes, such as proliferation, colony formation and invasion. Furthermore, high CTGF expression was associated with a spindle-like morphology whereas relatively low expression was associated with a cuboidal morphology. CTGF is secreted into the serum, so we performed a prospective study to predict tumor appearance. Surprisingly, the rats with high serum CTGF developed malignant mesothelioma much earlier than those with low serum CTGF and the difference was statistically significant. Therefore, I believe that CTGF can be a good target not only for the early diagnosis of malignant mesothelioma but also as a therapeutic target.

Finally, the surface of asbestos works as a natural niche for the chemical reactions of various molecules and we propose that asbestos may have provided environments for the origin of life with plasma, UV, radiation or other agents. A more detailed review on asbestos-induced mesothelial carcinogenesis is provided elsewhere.

### Risk of multi-wall carbon nanotubes

Carbon nanotubes are a synthetic material made only of carbon, first reported by Sumio Iijima. Because this material is resistant to heat, acid and friction but shows a high conductivity of heat and electricity with a flexible fine fibrous structure, it is already in the industrial market as battery cell and liquid crystal contents in solids. However, starting from the 2000s there has been concern about its mesothelial carcinogenicity, especially for multi-wall carbon nanotubes (MWCNTs) because its physical dimensions and characteristics are similar to those of asbestos.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Similarities and differences in Fe-NTA induced renal cell carcinoma and asbestos/carbon nanotube-induced mesothelioma in rats</th>
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<tr>
<td><strong>Fe-NTA-induced renal cell carcinoma</strong></td>
<td><strong>Asbestos-induced mesothelioma</strong></td>
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<tr>
<td><strong>Similarities</strong></td>
<td></td>
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<tr>
<td>Origin</td>
<td>Renal proximal tubular cell (mesodermal)</td>
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<tr>
<td>Iron</td>
<td>Local iron deposition in the target cells*</td>
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<td>8-OHdG/HNE-modified proteins (oxidative stress)</td>
<td>Homozygous deletion of p16/p15 tumor suppressor genes (33%)</td>
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<td>Genetic alteration in cancer</td>
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<td><strong>Differences</strong></td>
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<tr>
<td>Genetic alteration in cancer</td>
<td>Hemizygous deletion of p16/p15 tumor suppressor genes and methylation of the promoter region (33%); c-Met amplification; Ptpz1 amplification</td>
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<tr>
<td>Epithelial mesenchymal transition (EMT)</td>
<td>Most of the cancers are recognized as carcinoma; sarcomatoid change rare</td>
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<tr>
<td>Incubation period (average)</td>
<td>1.5 years</td>
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</table>

CNT, carbon nanotube; Fe-NTA, ferric nitrilotriacetate; HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine. Refer to text for details. [Corrections added on 7 March 2016, after first online publication: In Table 3, some realignments were made to the columns of the data. These have now been corrected and are indicated by the symbol *.]
We showed that MWCNTs have different carcinogenic abilities, depending on its diameter. Of note, MWCNTs with a 50 nm diameter showed potent carcinogenicity,\textsuperscript{132} which became the basis for the declaration by IARC that MWCNT with a 50 nm diameter is possibly carcinogenic to humans (Group 2B),\textsuperscript{133} together with previous results using p53 heteroknockout mice\textsuperscript{134,135} or tunica vaginalis of Fischer-344 rats.\textsuperscript{136} The characteristics of MWCNT with a 50 nm diameter are high crystallinity with rigidity, piercing mesothelial cells with a needle-like linear structure, which contrasted with other MWCNTs with a different diameter. Especially, MWCNT with a 15 nm diameter formed a tangling globule-like structure, which did not allow for their uptake/penetration by mesothelial cells with little mesothelial cytotoxicity and no mesothelial carcinogenesis.\textsuperscript{137} Mesothelial cell injury with in vitro experiments and peritonitis with experiments by single intraperitoneal injection after 4 weeks are in proportion to their carcinogenicity, and this simple method is effective in evaluating novel fibrous nanomaterials for their mesothelial carcinogenicity.\textsuperscript{132} We are proposing that these tests be used for assessing the mesothelial carcinogenicity of novel fibrous nanomaterials. Single-wall carbon nanotubes (SWCNTs) are recognized as non-carcinogenic to mesothelial cells thus far.\textsuperscript{138,139}

Of note, genetic alterations in malignant mesothelioma induced in rats by MWCNTs revealed almost identical genetic alterations as asbestos. Homozygous deletion of p15/p16 tumor suppressor genes was the most prominent,\textsuperscript{132} indicating that a pathology similar to asbestos works in MWCNT-induced mesothelial carcinogenesis, namely an excess iron exposure to mesothelial cells (Figs 5, 6). We observed iron deposition in close proximity to exposure sites of MWCNT, and furthermore, histones, hemoglobin and transferrin are adsorptive to pristine MWCNTs.\textsuperscript{140} Table 3 describes similarities and differences between Fe-NTA-induced renal cell carcinoma and asbestos/ MWCNT-induced mesothelioma. Excess iron in the target cells was in common, which appears to have induced a common genetic alteration, homozygous deletion of p15/p16 tumor suppressor genes. Early inflammatory responses by neutrophils were lacking for MWCNT.\textsuperscript{141}

**Non-thermal plasma as novel cancer therapeutics**

In the last ten years, a very exciting movement has occurred to apply non-thermal plasma to medical instruments and therapeutics.\textsuperscript{142–144} Plasma is the fourth condition of physical states out of the normal solid/liquid/gas phase and is a mixture of gas/radicals/electrons/cations/anions/UV. Plasma is represented by the sun in space, so it emits an immense amount of energy. However, recently, non-thermal atmospheric plasma was introduced (Fig. 7).

This is indeed a novel research area, but it is becoming increasingly clear that we can load the cells with adjustable near-natural oxidative stress with a much milder degree than radiation. Many chemical species have already been detected with non-thermal plasma exposure, including hydroxyl radicals, hydrogen peroxide, superoxide, nitric oxide, electrons and UV. We believe that the major species are hydroxyl radicals and UV.\textsuperscript{145} One of the problems in this area is that a standardization of the apparatus has not been established yet. In spite of these situations, there is sufficient data to reveal that non-thermal plasma works to promote wound healing\textsuperscript{146} and possibly cancer cell killing.\textsuperscript{147,148} The theoretical basis is based on the fact that cancer cells are generally oxidatively stressed.\textsuperscript{156} Therefore, additional oxidative stress may kill cancer cells, but not non-cancer cells. Furthermore, media exposed to non-thermal plasma appears useful for many applications, including age-related macular degeneration in the retina\textsuperscript{149} and cancer therapy.\textsuperscript{150} Not only hydrogen peroxide but novel species also

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appear to work in these situations, and intensive studies are now in progress.

CONCLUSION

I have been involved in the study of oxidative stress to clarify its biological effects for three decades. Of note, I, together with collaborators, developed two monoclonal antibodies to evaluate oxidative stress in formalin-fixed paraffin-embedded tissue specimens, which produced numerous fruitful results. I believe that oxygen, iron and food are three major components that drive our lives. Among these, iron is a persistent but targetable component as an initiator of oxidative stress. Excess iron is most likely working as the bottom of an iceberg for most carcinogenesis from Fenton reaction to tar.

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DISCLOSURE STATEMENT

None declared.

REFERENCES

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