BioScience Trends

International Research and Cooperation Association
for Bio & Socio-Sciences Advancement

Kyoto University Clock Tower Centennial Hall

ISSN 1881-7815 Online ISSN 1881-7823
Volume 2, Number 6, December 2008
www.biosciencetrends.com

BioScience Trends

IRCA BSSA

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BioScience Trends is a peer-reviewed international journal published bimonthly by *International Research and Cooperation Association for Bio & Socio-Sciences Advancement* (IRCA-BSSA).

BioScience Trends publishes original research articles that are judged to make a novel and important contribution to the understanding of any fields of life science, clinical research, public health, medical care system, and social science. In addition to Original Articles, BioScience Trends also publishes Brief Reports, Case Reports, Reviews, Policy Forum, News, and Commentary to encourage cooperation and networking among researchers, doctors, and students.

Subject Coverage: Life science (including Biochemistry and Molecular biology), Clinical research, Public health, Medical care system, and Social science.

Language: English
Issues/Year: 6
Published by: IRCA-BSSA
ISSN: 1881-7815 (Online ISSN 1881-7823)

Editorial Board

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Editors:

(*as of November 30, 2008*)

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Cover Photo of this issue

Kyoto University Clock Tower Centennial Hall

One of the most recognizable structures in Kyoto University campus is the Clock Tower, which was designed by Goichi Takeda, the first Professor of Architecture in this university, and completed in 1925. The Clock Tower designed using High-Tech at that time has been ticking for more than eighty years as a symbol of Kyoto University aiming the most advanced science and technology.

(*Photo by Jing Zhao*)

News

2008 Beijing Symposium on a Hearing Screening Program for Neonates and Children: Perspectives on interdisciplinary and international collaboration

Lihui Huang[1], Ruoyan Gai[2]

Keywords: Audiology, Hearing screening program, Neonates, Children, China

On December 27, 2008, the Beijing Symposium on a Hearing Screening Program for Neonates and Children was held at Beijing Tongren Hospital in Beijing, China. The symposium was sponsored by the Otorhinolaryngology Head and Neck Surgery Branch of the Chinese Medical Association and organized by the Beijing Otorhinolaryngology Academy, the Otorhinolaryngology Faculty of Capital Medical University, and Beijing Tongren Hospital of Capital Medical University.

More than 130 delegates from 34 medical institutions nationwide participated in the symposium. Dr. Demin Han, President of Beijing Tongren Hospital, and Dr. Xingkuan Bu, Leader of the Otorhinolaryngology Head and Neck Surgery Branch of the Chinese Medical Association, delivered opening speeches. Presentations at the symposium were given by Dr. Kimitaka Kaga from Japan's National Institute of Sensory Organs, Dr. Wei Tang from the University of Tokyo, Dr. Daofeng Ni from Peking Union Medical College Hospital, Dr. Xingkuan Bu from the First Affiliated Hospital of Nanjing Medical University, Dr. Zhiwu Huang from the People's Hospital of Wuhan University, Dr. Zhensheng Chen from China's Rehabilitation Research Center for Deaf Children, Dr. Xingqi Li from the Institute of Otolaryngology of the People's Liberation Army General Hospital, Dr. Yisheng Qi from the Beijing Institute of Otolaryngology, and Dr. Shusheng Gong from Beijing Tongren Hospital.

A hearing screening program for neonates and children is an effective way to provide early detection, diagnosis, and rehabilitation for hearing impairments. The program will also help with the development of appropriate speech, language, and cognitive abilities in hearing impaired children. Such a program has been suggested and implemented in developed regions like the US, Europe, and Japan since the 1990s and was implemented in China in 2002. In China, the incidence of a hearing impairment among infants is estimated to be 0.1-0.3%, indicating of an average of 20 million newborns each year 20-60 thousand will be born with a hearing impairment. Individuals with a hearing impairment account for the largest proportion of the disabled. The Beijing Symposium focused on implementation of a hearing screening program and early detection and interventions for

Figure 1. 2008 Beijing Symposium on a Hearing Screening Program for Neonates and Children.

Figure 2. Rehabilitation center affiliated with Beijing TongRen Hospital.

children with a hearing impairment in China. Experts presented the latest results in their respective fields and discussed research topics and future academic development. Topics covered included early hearing tests, methodologies of hearing screening programs, hearing and balance disorders in children, perspectives on infant audiology, and policy and planning aspects of hearing impairment management and rehabilitation.

Dr. Kaga has worked at the clinical forefront of hearing impairment detection and intervention for more than 30 years in Japan. This was his sixth visit to China. He described the hearing screening program in Japan and previous studies on hearing and balance disorders in children. Dr. Tang presented an explanation of study design, ethical issues, and contributions to clinical research and he affirmed the need for interdisciplinary and international collaboration in academic development. After the symposium, Dr. Kaga was accompanied by Dr. Tang and a reporter on a visit to a rehabilitation center affiliated with Beijing Tongren Hospital and Dr. Kaga consulted on the case of a seven-month-old infant with bilateral hearing impairment and his family.

At the end of the Symposium, Dr. Han emphasized the importance of hearing impairment management and audiological development. Implementation of a consistent hearing screening program for neonates and children involves various fields such as otorhinolaryngology, audiology, pediatrics, maternal and child care, and social medicine. The Symposium succeeded in broadening horizons with regard to effective detection, diagnosis, treatment, and rehabilitation of children with a hearing impairment in China and it highlighted the importance of interdisciplinary and international collaboration in these efforts. (*Reported on Dec. 27, 2008*)

Appendix

- Progress of researches on hearing and balance disorders in children. (*Kimitaka Kaga, National Institute of Sensory Organs, National Tokyo Medical center, Tokyo, Japan*)
- Study design and ethical issues for clinical and basic research. (*Wei Tang, The University of Tokyo Hospital, Tokyo, Japan*)
- Academic and policy issues on early detection of hearing impairments in infants. (*Daofeng Ni, Peking Union Medical College Hospital, Beijing, China*)
- Development of infant audiology in China. (*Xingkuan Bu, The first affiliated hospital of Nanjing Medical University, Nanjing, China*)
- Clinical practice of early detection and intervention programs for children with a hearing impairment. (*Zhiwu Huang, Renmin Hospital of Wuhan University, Wuhan, China*)
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- Closing remarks. *(Xingqi Li, Institute of Otolaryngology, Chinese PLA General Hospital, China*)

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Review

Inflammaging (inflammation + aging): A driving force for human aging based on an evolutionarily antagonistic pleiotropy theory?

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Summary **Aging, and especially human aging, can be explained by the emerging concept of para-inflammation-driven inflammaging, *i.e.* a combination of inflammation and aging. Inflammaging posits that aging either physiologically or pathologically can be driven by the pro-inflammatory cytokines and substances produced by the innate immune system. Animals must maintain homeostasis as they age despite incessant attack from both intrinsic and extrinsic stimuli/antigens. These potentially harmful pro-inflammatory signals at a later stage of life may act antagonistically to the beneficial role they had in an earlier stage of life, like serving as developmental engines for body system formation. The concept of inflammaging is based on an antagonistic pleiotropy theory programmed during evolution. Clinical trials including caloric restriction, sirtuin activators, and p38 MAPK inhibitors against both pathological aging such as metabolic syndrome, diabetes mellitus, rheumatoid arthritis, and Werner syndrome and physiological aging have been proposed.**

Keywords: Aging, Antagonistic pleiotropy, Caloric restriction, Cytokine, Evolution, Inflammaging, Inflammation, Innate immunity, Metabolic syndrome, Rheumatoid arthritis, Sirtuin, Werner syndrome

1. What is aging?

Bernard Strehler proposed the classical definition of aging 30 years ago (*1*) according to the following four characteristics. Although these criteria are generally accepted, recent progress in the fields of evolutionary science, gerontology, and inflammation research in a variety of models have led to slight modifications of this view, as are noted in comments here:

1. Universality: Changes should occur in all older members.
2. Intrinsicality: Aging does not result from modifiable, environmental variables.
   Comment: Recent experiments suggest the significantly modifiable effect of environmental factors such as caloric restriction on aging in a variety of species (*2-6*).
3. Progressiveness: Aging begins with a gradual and cumulative occurrence of onset like development and maturation.
   Comment: Aging begins immediately after maturity. The basic mechanism of aging may different from development and maturation, which are heavily controlled genetically. Restoration and repair of decreased function and damaged tissue can be interventionally obtained, for example, by caloric restriction (*2-7*).
4. Deleteriousness: The most characteristic change that differentiates aging from development and maturation is its deleteriousness; minute, but progressive decline of the whole aspect of physiological functions in a concerted fashion.
   Comment: As the term 'aging' basically means concerted changes in an organism over time and does not solely mean senescence with weakness, aging should include all stages of an organism's life: development, maturation, and senescence from birth until death. Some physiological functions such

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as those of the nervous system and psychological system may remain unchanged if pathological dysfunctions can be prevented or repaired (*8-15*). However, most functions/systems in the human body tend to deteriorate slowly and progressively with age like machines, and the terms "aging" and "senescence" are used interchangeably even in the field of gerontology.

Human aging has been viewed as the declining function of most body systems as the result either of the progressive accumulation of damaged tissue and substances or the progressive loss of normal tissue and substances either by intrinsic or extrinsic mechanisms. As no older individuals can escape from age-related pathologies like atherosclerosis, osteoporosis, insulin resistance, and sarcopenia, defining the elderly as healthy is problematic. In practice, elderly who live independently in relatively good health are considered healthy or normal, even if they have a certain degree either of hypertension, osteoporosis, sarcopenia, insulin resistance, cognitive dysfunction, or other age-related organ dysfunctions according to standards for healthy young adults. Thus, the term "successful aging or better aging" has been proposed. Theoretically, aging can be divided into two categories: physiological (normal/intrinsic) aging and pathological (diseased/extrinsic) aging, though a clear-cut separation between the two is difficult.

The four major theories on the mechanisms of aging that have been proposed are listed in Table 1.

1. Wear & tear theory was the historically accepted theory; all living things that exist under the control of time may be damaged either by extrinsic environmental damage such as radiation, free radicals, infections, and attacks from the predators or by intrinsic environmental damage such as free radicals and metabolites. This damage may induce somatic mutations leading to malignancy: the acceleration of aging. This mechanism may not be genetically inherited.
2. Wear & repair theory has been proposed as a variation of wear & tear theory. This theory suggests the mechanisms of inheritable maintenance and repair.
3. Mutation accumulation theory is widely supported by oncologists and cell biologists and includes the idea that oncogene-induced senescence is part of the barrier to tumorigenesis (*16,17*).
4. Antagonistic pleiotropy theory has been favored in the form of the term "evolutionarily antagonistic pleiotropy."

2. Evolutionarily antagonistic pleiotropy

Natural selection that includes a "struggle for existence" and "survival of the fittest" as proposed in evolutionary theory has been widely accepted as the basic driving force leading to major biological changes (*18,19*). Aging has generally been believed to be time-dependent reduced Darwinian fitness resulting in a growing risk of disease and death that all living things inevitably experience in the later stage of life. It is also usually accompanied by a decline in fertility. Historically, aging has never been welcomed, especially among evolutionists, as they had difficulty in finding the merit of aging as an organism (*20*).

The advantages of programmed aging may be that:

1. It benefits the species/groups by preventing overcrowding and/or facilitating further evolution by securing a turnover of generations (*21*). This theory may explain the benefit of the species or group but supersedes the contrary interests of the individuals (*22*).
2. Aging stops cells that have escaped from normal control from dividing indefinitely, such as cancer and atherosclerotic plaques as cited by cell biologists (*23-25*).
3. Aging-associated declining function/metabolism with low/no fertility may provide benefits under stress such as starvation (*26,27*).

According to conventional evolutionary theory, aging as a non-adaptive process has been explained as the result of the weakening force of natural selection and reduced genetic effects with age (*28,29*).

This theory offered the following explanations for the apparent disadvantage to the individual:

1. Detrimental mutations acting only post-maturity may not be a reproductive disadvantage to individuals and thus can spread through populations.
2. Although the risk of death that individuals experience from environmental attack can increase with aging,

Table 1. The major mechanistic theories of aging

Theory	Cause of aging	Inheritance
1. Wear & tear	Radiation, Free radicals, Mechanical damage, Somatic mutation	No
2. Wear & repair	Radiation, Free radicals, Mechanical damage, Somatic mutation	Yes/No
3. Mutation accumulation	Deleterious mutation late in life	Yes
4. Antagonistic pleiotropy	Genes with beneficial effects early in life and detrimental effect later	Yes

the individual's genes can be inherited and spread to the next generation and the chance of evolutional mutations may be enhanced before senescence by fragility and death.

Recent reports on wild red deer and sheep suggested genotype-by-age interactions (age-specific additive genetic covariance matrices across all ages without the dramatic loss of power associated with subdividing the data into age classes), which the evolutionary theory of aging already predicted. However, an increasing genetic variation with aging (additive genetic variation in individual aging rates) was observed (*30*,*31*).

According to antagonistic pleiotropy theory, the effects of aging should be beneficial in early life, when natural selection is strong, but harmful in later life, when selection is weak. Antagonistic pleiotropy theory in regard to the evolution of aging predicts that increased early-life performance should be accompanied by earlier (or faster) aging (*27*,*32*,*33*). Quantitative genetic pedigree analyses in wild swans indicate that traits from groups at the age of first reproduction and at the age of last reproduction had additive genetic variance, but both were positively genetically correlated (*34*). Thus, both traits show heritable variation and are under opposing directional selection, but their evolution may be constrained by a strong evolutionary trade-off. These results are consistent with the theory that increased early-life performance comes with faster aging because of genetic trade-offs.

Although Csete *et al*. proposed against evolutionary antagonistic pleiotropy in that the enormous complexity of organismal systems is the result of an evolutionary trade-off between robustness, feedback, and fragility (*35*), the randomness of the evolutionary process may retain the characteristics of antagonistic pleiotropy: beneficial events in earlier stages despite their later potential drawbacks. Evolution may not see into the future (*36*,*37*).

However, recent papers have proposed a new concept, programmed and altruistic aging (*38*). They proposed two altruistic reasons for the evolutional advantage of aging and death:

1. Programmed and altruistic aging benefits closely related organisms that have acquired mutations that increase their ability to grow and survive.
2. Programmed and altruistic aging benefits the species/group as a whole.

Recent studies of unicellular organisms have raised the controversial possibility that programmed and altruistic aging might occur, and that this might be an adaptive process that benefits small sub-populations of closely related mutants (*39-41*).

Evolutionary theory has not dealt primarily with individuals but with population/species using the concept of natural selection as a driving force for the "struggle for existence" and "survival of the fittest" (*18*,*19*). Although aging is a nearly universal feature of multicellular organisms, aging, and especially human aging, involves a purely individual phenomenon occurring after the reproductive stage and may involve freedom from the forces of natural selection.

Evolutionary theory with regard to aging is still in the formative stages because experimental evidence on aging, and especially among human beings, may be difficult to obtain. In addition, aging may not be a simple straight road to longevity or the end of life; aging may also fine tune organismal systems in a sophisticated concerted manner with a 'longevity' gene(s) and environmental stress, as was observed in the coagulatory systems of centenarians (*42*,*43*). Aging may be a new battlefield for evolution.

3. What is inflammation?

Inflammation, triggered by harmful stimuli and agents like infection and tissue injury, is defined as a wide variety of adaptive physiological and pathological processes to avoid infection and repair damage, restoring the organism to the usual state of homeostasis (*44*).

As Medzhitov mentioned in his review article in Nature, pathological aspects of many types of inflammation, either of acute or chronic, have been well documents, though most physiological functions of inflammation have not been fully studied (*45*). He explained three modes of adaptation and maintenance of tissue/cell homeostasis in relation to inflammation.

1. Under baseline conditions, tissues/cells maintain homeostasis. Apoptosis is a type of homeostatic mechanism during development, growth, and also aging.
   However, recent study of lymphoid tissue genesis induced by bacterial flora commensals through innate receptors suggests a countercurrent modification of homeostasis (*46*).
2. If damaged or infected, tissues/cells respond & repair as a result of acute/chronic inflammation.
3. If conditions waver in between homeostasis and infection, such as mild/slowly progressive stress or modest malfunction, the tissue/cells tend to finely adapt to the slightly changed conditions and restore tissue/cell functionality by inducing para-inflammation, sub-inflammation, low-level of inflammation, sterile inflammation, physiological inflammation, or inflammaging as Franceschi proposed (*47*).

Dysregulated para-inflammation may be responsible for mild chronic inflammatory conditions in age-related diseases like insulin resistant type II diabetes mellitus, atherosclerosis, cancer, and Alzheimer's disease (*48-52*).

Traditional evolutionary theory predicts the existence of genes with antagonistic functions on development, maturation, and aging.

4. What is inflammaging?

The term "inflammaging" is a coinage of "inflammation" and "aging" by Italian immunology researchers (*47,53*). For the immune system, the characteristic consequence of aging, they posited, is the progressive filling of the immunological system by activated lymphocytes, macrophages, and dendritic cells in response to chronic/continuous subtle stress either from pathological or physiological antigens/toxins. Thus, the condition of inflammaging provides a continuous mild antigenic challenge leading to a pro-inflammatory condition associated with the progressive stimulation/depletion of the immune system and other organismal systems (*14,29,53,54*). On the whole, immunosenescence can be taken as proof that the beneficial effects of the immune system, devoted to the neutralization of dangerous/harmful agents early in life and to better development and maturation leading to the prosperity of future generations and species in adulthood, become detrimental late in life, in a period largely not foreseen by evolution (*37*). This perspective fits with basic assumptions of evolutionarily antagonistic pleiotropy theory in regard to aging, which suggests that a trade-off between early beneficial effects and late negative outcomes can occur at the genetic and molecular level.

Inflammaging can be defined by:

1. Low-grade.
2. Controlled.
3. Asymptomatic and not pathological.
4. Chronic.
5. Systemic inflammatory state (*54*).
6. Beneficial effects in early life but detrimental effects in later life for individuals.

Although several groups from different research backgrounds have studied the inflammatory process in human aging (*55-58*), the inflammaging theory of human aging fails to clearly explain the "true" physiological aging process as a whole, despite a fairly large amount of evidence for pathological aging associated with the natural aging process (*48-52,59*). This is because of difficulty in separating "true" physiological aging and "true" pathological aging during the natural aging process, as mentioned in the previous section.

The contribution of inflammatory/infectious processes to the pathogenesis of age-associated diseases ("true" pathological aging) has been frequently discussed in terms of atherosclerotic cardiovascular diseases (*60-64*). Although atherosclerotic cardiovascular diseases such as myocardial infarction and cerebral bleeding are unquestionably diseases that are closely related to the aging process, one would be hard-pressed to distinguish whether atherosclerosis itself, and especially at the sub-clinical level, is the "true" pathological process of age-associated disease or just the accumulation of subtle but continuous physiological processes of natural aging. In the pre-clinical stage, other aging-associated diseases such as osteoporosis, type II diabetes mellitus, sarcopenia, osteoarthritis, Alzheimer's disease, and hypogonadism are also difficult to clearly differentiate as a diseased state or a non-normal state.

This difficulty may exist because of the following reasons:

1. Individuals develop, mature, and successively age with senescence after being born as an infant, and individuals maintain the consistency of their own systems as an organism throughout their lives.
2. According to the traditional point of view, diseases are an abnormal state that is detrimental to one's quality of life or that cannot be readily survived without medical intervention.
3. In modern society, a "diseased state" may not be apparent in an individual and extremely sophisticated medical examinations can easily detect minute changes in bodily constituents that may not impact the individual's life in the immediate future. And yet a doctor may consider the individual to have a "disease". However, a "diseased state" or an accumulation of physiological aging may be a prologue to age-associated diseases leading to an organism's death in the distant future.
4. Thus, distinguishing between a non-normal state and a true diseased state, and especially one that is age-associated, is difficult.

5. Clinical trials of drugs to treat pathological inflammaging

Several suggestions have been provided by clinical trials using anti-inflammatory interventions. The first evidence was reported in the field of atherosclerosis (*48,49,65*). A low dose of aspirin can prevent angina pectoris and myocardial infarction and extend the life-span of the patient. In addition, the important point is that intervention can significantly reduce the risk of cardiovascular disease even in apparently healthy individuals. This study suggests the potentially modifiable role of medical intervention in physiological aging in addition to that in pathological aging.

In cancer treatment, targeted inhibition of the cyclooxygenase-2 pathway may be effective in the treatment of colorectal cancer and other types of cancer through a reduction in tumor-associated inflammation (*66*). Osteoporosis, characterized by low bone mass and increased bony fragility, is not recognized as a disease

prior to an examination of bone mineral density or actual fracture. A recent study suggests an influential effect of inflammation on the occurrence of osteoporosis (*50*). Non-steroidal anti-inflammatory agents were found to have a protective with respect to the development of Alzheimer's disease (*51,67*), though a disagreement with this finding was also reported (*68*).

Caloric reduction is another type of anti-inflammatory intervention for aging and type II diabetes mellitus (*2-7,59*). In addition, the successful treatment of insulin-resistant diabetes mellitus by several pharmacological interventions has been reported in inflammatory pathways such as pioglitazone, high-dose aspirin, PPARα and γ ligands, and anti-TNFα (*69-71*).

6. What drives inflammaging?

The potential forces that drive inflammaging are pro-inflammatory cytokines and substances as are listed in Table 2. CRP and fibrinogen, the major clinical markers of inflammation, have been significantly associated with coronary disease, myocardial ischemia, and myocardial infarction, in association with IL-1, IL-1 receptor antagonist, IL-6, soluble IL-6 receptor, IL-18, TNFα, serum amyloid A, and soluble ICAM-1 (*56,72-74*).

Table 2. Associated changes of inflammation with aging

Agents	Inflammation	Aging
Inflammatory proteins		
CRP	↑*	↑
SAA	↑	↑
Proinflammatory mediators		
IL-1α, β	↑	↑
IL-4	↑	?***
IL-6	↑	↑
IL-12	↑	↑
IL-15	↑	↑
IL-18	↑	↑
TNFα, β	↑	↑
IFNγ	↑	↑
TGFβ1	↑	↑
sIL-2R	↑	↑
sIL-6R	↑	↑
MCSF	↑	↑
GM-CSF	↑	↑
Chemokines		
IL-8	↑	↑
MCP-1	↑	↑
Anti-inflammatory mediators		
IL-1ra	↑	↑
sTNFR	↑	↑
IL-10	↑	↓
Proinflammatory enzymes		
iNOS	↑	↑
COX2	↑	↑
PGE2	↑	↑
Adhesion molecules		
ICAM-1	↑	↑
VCAM-1	↑	↑
Hypoxic markers		
HIF-1α	↑	↑
VEGF	↑	↑
Redox state		
ROS	↑	↑
SOD	↓**	↓

* ↑, increased; ** ↓, decreased; *** ?: conflicting results.

Cancer-related inflammation is reported under the conditions described below (*75,76*):

1. Growth of tumor cells associated with leukocyte recruitment and survival is initiated by G-CSF, GM-CSF, M-CSF, TGF-β, PDGF, IGF-1 and bFGF.
2. Monocyte recruitment and angiogenesis are activated by chemokines such as IL-8, CC-chemokine ligand 2/MCP-1, and CCL20 and modulated by IL-4 and IL-12.
3. Tumor cell homing to lymph nodes is promoted by chemokines, chemokine receptors, and adhesion molecules including CXC-chemokine receptor 4, CXC ligand 12, and L-selectin.
4. Tumor cell invasion and dissemination may be promoted by proteases including MMPs 7, 9, and 10 and urokinase-type plasminogen activator.
5. Fibrosis as the result of tissue repair was accelerated by TGF-β, PDGF, IL-1, IL-4, and mast cell tryptase.

However, whether an age-associated "pro-inflammatory condition" is the result of the primary impairment of the mechanisms that induce the inflammatory response or is the net result of cardiovascular risk factors including smoking, obesity, alcohol consumption, sedentary lifestyle, and excessive stress is still unclear (*74*).

Several lines of basic research suggest the important roles of inflammation and perhaps chronic infection in the initiation and progression of atherosclerosis (*5,44,47,49,61*). For example, prior exposure to *Chlamydia pneumoniae*, cytomegalovirus, and *Helicobacter pylori* has been detected in atherosclerotic tissue in humans (*62,77-81*). Cytomegalovirus infection can also induce atherosclerosis with endothelial lesions in animal experiments (*63,82*). These findings suggest that these microorganisms may activate vessel-associated leukocytes or lymphocytes or induce the transformation of vascular muscles or vascular endothelial cells (*81*).

As summarized in Table 3, an association between inflammation/infection and cancer risk has been proposed (*76,77,82,83*) and the successful prevention and treatment of colon cancer both in humans and mice by cyclooxygenases and stomach cancer by

Table 3. Cancer risk associated with infections

Cancer	Infections
Baldder/colon cancer	*Schistosoma haematobium*
Cervical cancer	Papilloma virus
Stomach cancer	*Helicobacter pylori*
MALT lymphoma	*Helicobacter pylori*
Hepatocellular carcinoma	Hepatitis virus B, C
Kaposi's sarcoma	Herpes virus
Nasopharyngeal carcinoma	Epstein Barr virus
Burkitt's lymphoma	Epstein Barr virus
Rous sarcoma	Rous sarcoma virus

antibiotics is widely accepted (*83-85*). Sarcopenia and frailty syndrome leading to accelerated mortality may be caused by the apoptotic death of muscle cells mediated by TNFα (*86*). Inflammatory cytokines including TNFα, IL-1, and IL-6 have been reported to be associated with cognitive decline with aging and Alzheimer's disease (*86*).

7. Innate immunity

The physiological aging process and many age-associated diseases are likely orchestrated with pro-inflammatory cytokines and chemokines by reactive oxygen species and reactive nitrogen species reactions through the activation of NFκB, which has a central position in the inflammatory reaction (*56,57*). However, what and how bodily defense systems including the immune system cope, modulate, and respond to a time-dependent environmental attack either from the inside or outside is still unclear.

The immune system operates in concert with two evolutionarily different branches: innate (natural) immunity and adaptive (acquired) immunity. The host immune system recognizes and differentiates between different inflammatory triggers such as tissue injury, bacterial/viral/parasitic infections, food, drugs, and mutant cells using specific receptors. Most microbial infection and some fragmented tissue products can be detected by innate immune receptors known as Toll-like receptors (TLR) on the surface of macrophages, polymorphonuclear cells, dendritic cells, and mast cells (*87-90*). Microbes and fragmented tissue products that may be recognized by TLR are listed in Table 4.

Cells dying as the result of asterile tissue injury such as ischemia-reperfusion or apoptosis during normal development can trigger an inflammatory cytokine response mimicking the features of infection-induced inflammation (*91-94*).

A decreased ability to maintain homeostasis in response to external stress in association with an increased risk of age-associated diseases and death has been studied in the elderly (*95,96*). At over 60 y.o., individuals have a mortality up to 25 times that of the individuals between 25 and 44 y.o.; when compared to the individuals between 25 and 44 y.o., specific mortality rates in people over 65 y.o. are much higher as a result of the following factors: ~90-fold for heart disease and pneumonia/influenza, 43-fold for cancer, and more than 100-fold for stroke and chronic lung diseases. As resistance to/defense against these age-associated pathologies may depend on the immune system, these data suggest that aging and innate immunity play a pivotal role of in controlling longevity of the elderly.

Accumulating evidence indicates the possible role of innate immunity-mediated inflammaging in human aging process.

However, there are still a number of unanswered questions (*97-100*).

1. How does the innate immune system recognize the degree of harmful inflammation that may lead to the following outcomes: homeostasis (complete repair), partial repair, modified repair, additional/collateral damage or death?
2. In addition, do qualitatively different types of insults to the host such as sterile tissue injury and infection produce similar inflammation since the ligands that lead to subsequent signals may follow similar innate immune pathways?
3. What are the mechanisms to resolve innate immunity-mediated inflammation as accompanies aging?
4. How do aging organisms confront, *via* innate immunity, the continuous attack of inflammaging and maintain a slightly changed/aged state?

According to the general theory of hormesis (*101,102*), the beneficial effects of extremely low doses of agents including those from apoptotic cells and fragmented matrix components during normal development and maturation are otherwise toxic at higher doses that can not be fully cleared from the tissues and cells, and accumulate with aging within the organism (*85,103-106*).

Several groups of researchers have suggested that inflammaging may be an auto-innate immunity subclinical syndrome induced by self-constituents released from apoptotic cells or degraded products during physiological development and daily tissue

Table 4. Toll-like receptor and ligands

TLR	Ligand
TLR1	Bacterial triacyl lipopeptides, Peptidoglycan, Lipoteic acid, Zymozyan, Hemagglutinin, Virus
TLR2 (cell surface)	Triacyl lipopeptides, Peptidoglycan, Lipoteic acid, Zymozyan, Hemagglutinin, Virus
TLR3 (endosome)	Viral dsRNA, Poly (I:C), Endogenous RNA from necrotic cells
TLR4 (cell surface)	LPS (gram(-)), Hsps, Hyaluronan, Fibronectin
TLR5	Bacterial flaggellin
TLR6	Bacterial diacyl lipopeptides
TLR7/TLR8 (endosome)	Viral ssRNA
TLR9 (endosome)	Bacterial/viral CpG DNA, Chromatin IgG complexes, HMGB1
TLR10	not yet identified
TLR11	not yet identified in humans

damage (*54,105,106*).

Evolutionary programming of the innate immune system leading to inflammation may act beneficially before maturation as a driving force for physiological development linked, for example, to apoptosis, but act detrimentally after maturation as a harbinger of both the physiological and pathological aging. Thus, this programming may act *via* evolutionary selection of these genetic traits.

8. Models of human inflammaging

Three different types of potential disease models for human inflammaging are 1) metabolic syndrome, 2) rheumatoid arthritis, and 3) Werner syndrome. The similar but accelerated clinical aspects of these diseases in response to natural aging are summarized in Table 5.

8.1. *Metabolic syndrome*

Metabolic syndrome consists of a combination of abdominal fat deposition, hypertriglyceridemia, low high density lipoprotein, hypertension, and fasting hyperglycaemia that can lead to diabetes mellitus and atherosclerosis (*96*). Diabetes mellitus has been proposed as a model for an accelerated form of human aging (*107-109*). Recent accumulated evidence suggests a pivotal role for inflammation in the pathogenesis of diabetes mellitus, obesity, and metabolic syndrome resulting from an overeating and inactivity in postindustrial societies (*2,4,57*). Obesity is closely associated with a series of sequentially appearing health problems including insulin-resistant type II diabetes mellitus, fatty liver, atherosclerosis, hypertension, neurodegenerative Alzheimer's disease, chronic obstructive lung disease, and even some cancers (*69*). As indicated in Table 5, some of the major clinical manifestations usually noted in the natural aging process such as secondary dwarfism, cataracts, and loss of hair are not usually encountered in metabolic syndrome. In addition, although the data on the aging-related immunological dysfunctions are, except for an elevation of pro-inflammatory cytokines, lacking, several endocrine-metabolic disorders in an accelerated fashion are consistent hallmarks of metabolic syndrome (*110-112*). Caloric restriction along with appropriate exercise has been suggested as an effective treatment for metabolic syndrome (*1-6,69,71*).

8.2. *Rheumatoid arthritis*

Rheumatoid arthritis is not usually recognized as a type of accelerated aging disorder (*113*). Patients with rheumatoid arthritis do not usually experience a higher incidence of cancer except lymphoma, but do exhibit a significantly higher incidence of atherosclerotic diseases, sarcopenia, sleep disorders, and osteoporosis in comparison to the general population (*114-117*). In spite of substantial recent medical progress, the average life-span of patients with rheumatoid arthritis remains far lower than that of general population (*118-121*). They show some signs of metabolic syndrome as listed in Table 5, though disease-specific therapy may contribute to the development of age-associated pathologies to a certain degree. In addition, immunological hallmarks usually observed in rheumatoid arthritis can be viewed as the result of an accelerated immunological state due to aging such as increased serum immunoglobulin levels, positivity for rheumatoid factor and anti-nuclear antibodies, increased

Table 5. Clinical aspects of inflammation-associated diseases

Signs & symptoms	Natural aging	Metabolic syndrome	Rheumatoid arthritis	Werner syndrome
Connective tissue disorder				
Dwarfism	+	+	++	++
Gary hair/alopecia	+	+	++	++
Skin atrophy	+	+	++	++
Sarcopenia	+	++	++	++
Arthropathy	+	++	++	++
Cataract	+	++	++	++
Osteoporosis	+	+	++	++
Endocrine-metabolic disorder				
Diabete mellitus	+	++	++	++
Hypogonadism	+	++	++	++
Hyperlipidemia	+	++	++	++
Hyperuricemia	–	++	++	++
Central obesity	+	++	++	++
Immune disorder				
Autoantibody production	+	+	++	++
Recurrent infection	+	+	+	+
Pro-inflammatory cytokines ↑	+	++	++	++
Neurodegenerative disorder	+	++	–	–
Cancer	+	++	++	++
Atherosclerosis	+	++	++	++
Hypertension	+	++	+	+

production of pro-inflammatory cytokines, decreased DTH reaction to BCG, and decreased production of IL-2 and γIFN (*113,122-124*). As recommended by the American College of Rheumatology, the standard treatment protocol for rheumatoid arthritis involves treating chronic autoimmune-mediated inflammation (*125,126*). Recent anti-TNFα therapy is reported to improve both age-related conditions such as insulin resistance and arthritic inflammation in patients with rheumatoid arthritis (*127*). Interestingly, the traditional use of hydroxychloroquine and sulphasalazine can improve insulin resistance in parallel with an improvement in condition (*128-130*).

8.3. *Werner syndrome*

Werner syndrome has been recognized as typical progeroid syndrome mimicking accelerated human aging (*131*). As listed in Table 5, patients with Werner syndrome manifest a wide variety of aging phenotypes immediately after maturity (*132-134*). Werner syndrome is an autosomal recessive disease involving mutation of the RecQ3 helicase and a shorter life-span (*134-137*). Dysfunction of the RecQ3 helicase, resulting in the unwinding of the double helices of DNA and RNA unquestionably leads to the typical Werner syndrome symptoms of metabolic syndrome (*131-133,138-140*). The major causes of death are myocardial infarction and cancer in concert with causes of death in the general population. A reason for interest in Werner syndrome is the constant presence of immunological and metabolic abnormalities that usually overlap normal aging (*141-146*). Although patients with Werner syndrome usually do not manifest apparent inflammatory symptoms such as recurrent infection and chronic inflammatory diseases, elevated serum levels of inflammatory cytokines including IL-6, TNFα, adipocytokines, and TGFβ have been frequently detected in addition to elevated levels of soluble Fas ligand, MMP1 and 9, hyaluronan, and fibronectin (*138-140,144,147,148,* unpublished data). There is no clear association between these inflammatory markers and age-related phenotypes in Werner syndrome except as regards diabetes mellitus. In patients with Werner syndrome, levels of plasma adipocytokines, *i.e.* significantly elevated TNFα and decreased adiponectin, can return to normal after treatment with pioglitazone (*139*).

9. Caloric restriction: Clues to support the concept of inflammaging as a form of evolutionarily antagonistic pleiotropy

Inflammaging is a low-grade (mild and subtle), controlled (easily adjusted to a homeostatic state), asymptomatic (not pathological or unrecognizable), chronic (near-steady state), and systemic inflammatory state. The concept of inflammaging coincides with antagonistic pleiotropy theory in regard to the evolution of aging, postulating that aging is the late deleterious effect of genes (pro-inflammatory *vs.* anti-inflammatory) that are beneficial at an earlier stage of life for the development and maintenance of body systems (*47*). Restriction of food intake (caloric restriction) can extend the maximum and average life span of laboratory animals by delaying natural aging processes (*2-6,149*). Although the evidence on an underlying mechanism of countering natural aging is scarce, a hypothesis, in line with hormesis theory (*101,102*), that links the alteration of glucose-IGF1 and growth hormone has recently gained support (*4,6*).

In addition, the crucial role of hypothalamic energy-sensing neurons in the control of energy metabolism-derived inflammation has been suggested (*4,6*). A recent paper by Sinclair indicated that resveratrol (3,5,4'-trihydroxy stilbene) is an effective drug for maintaining the health and extending the life span of laboratory mice (*150*). Resveratrol, a small polyphenolic SIRT1 activator found in red wine, can modulate energy metabolism by enhancing insulin sensitivity, decreasing plasma IGF-1 levels, increasing AMP-activated protein kinase and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) activity, and increasing the number of mitochondria with improved motor function (*150*). Resveratrol, a sirtuin activator, has been implicated in several important cellular processes, including DNA repair, p53-mediated apoptosis, and adipogenesis and is reported to extend life-span in many animal models like those involving caloric restriction (*150-153*). With the birth of life on Earth millions of years ago, life had to evolve to maximize its metabolic efficiency to obtain as much energy as possible in a severely nutrient-scarce environment. Thus, life must acquire better metabolic systems during evolutionary processes. Consequently, an organism can accumulate an excess amount of fat/energy resources in a relatively rich environment like in today's postindustrial societies (*2,4,6*). A "hyper-adapted" system of energy metabolism (based on the 'thrifty gene' hypothesis) (*154*) may act beneficially as a driving force for physiological development and maturation but may induce and accelerate aging after maturity through inflammaging as a result of natural selection (*96*).

10. Future perspective

Along with the recent concept of linking inflammaging to innate immunity to explain aging, several lines of anti-inflammatory intervention such as caloric restriction, SIRT1 activators, and p38 MAPK inhibitors for diabetes mellitus, sarcopenia, arthritis, and life-extension have been proposed for a variety of species (*2-7,71,150,155-158*). However, the relatively complex functional mechanisms of the SIRT1/SIR2 pathway that

regulate the p38 MAPK, p53, and energy metabolism pathways require extensive study in future clinical trials (*159-162*).

Science has developed and progressed based on strictly logical evidence according to current standards. Although science demands a rational answer for every phenomenon, such inquiry is irrelevant to most animals, and current scientific standards fail to explain why they experience decay and death.

"Aging", a term for a mechanism of inflammaging based on antagonistic pleiotropy theory, may represent a transitional indicator of unceasing evolutionary dynamics. Neither man nor even evolutionary theory can unfalteringly predict the future.

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(*Received December 18, 2008; Accepted December 29, 2008*)

Brief Report

Adenovirus-mediated siRNA inhibited survivin gene expression induces tumor cell apoptosis in nude mice

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Summary **In order to research the survivin gene's action on an animal tumor, we used an adenovirus-mediated siRNA system to inhibit the expression of survivin in an animal model of hepatocarcinoma using nude mice. We constructed a hepatocarcinoma model with nude mice using the hepatocarcinoma cell line HepG2 and divided the mice into four groups depending on the injection dose of AdsiRNA-survivin. We injected the constructed survivin-siRNA adenovirus into tumor-bearing nude mice, observed tumor growth, and determined the tumor growth curve. We then detected tumor cell apoptosis using a TUNEL kit that can assay sliced DNA in tumor cells. The growth of tumors injected with a high or low dose of AdsiRNA-survivin was obviously inhibited, and this level of inhibition was positively correlated with the injected dose of adenovirus. Results of the TUNEL test showed that many of the apoptotic cells were brown in color with concentrated nuclei and an irregular cell shape for both the high and low injection doses. The number of apoptotic cells decreased by group in the order of the high dose group, the low dose group, the AdsiRNA-U6 group, and the PBS group. In conclusion, our results demonstrated that an adenovirus-mediated siRNA system can be used for animal experiments *in vivo*. AdsiRNA-survivin efficiently inhibited tumor growth and induced tumor cell apoptosis, and it did so in a dose-dependent manner.**

Keywords: Adenovirus-mediated siRNA, Survivin, Nude mouse model, *In vivo*, Apoptosis

1. Introduction

Tumors are a serious threat to human health. Every year in China, over 2.5 million people are diagnosed with cancer and more than 150 billion RMB is spent to treat over 6 million patients. At present, treating tumors with gene therapy is a focus of biomedicine, as inhibitors of programmed cell death (apoptosis) aberrantly prolonging cell viability may contribute to cancer by facilitating the insurgence of mutations and by promoting resistance to therapy.

Survivin is a recently discovered inhibitor of apoptosis (IAP) (*1*) that obviously counteracts apoptosis and is highly conserved between different species. The full length of the survivin gene is 14.7 kb, coding a 16.5 ku cytoplasmic protein including 142 amino acids. In order to research survivin's action on mammal tumors by RNAi *in vivo*, an animal model of hepatocarcinoma was created using nude mice and a recombinant adenovirus named AdsiRNA-survivin (adenoviral vector-mediated siRNA of the survivin gene) was constructed with highly efficient infectivity and tumor inhibition (*2*). Survivin gene expression was detected in hepatocarcinoma cells and the growth of transplanted human hepatocarcinoma was obviously inhibited by AdsiRNA-survivin *in vivo*, as indicated here, since many of the human hepatocarcinoma cells were apoptotic.

2. Materials and Methods

2.1. *Plasmids, adenovirus, cell lines, and nude mice*

The plasmids pAdTrack and pAdEasy-1 were provided by Dr. Tong-Chuan He of the University of Chicago Medical Center, USA. The adenovirus

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vector pAdsiRNA-survivin and pAdU6-control and the recombinant adenovirus AdsiRNA-survivin and AdU6-control were constructed at this lab; adenovirus titers were 2.4×10^9 pfu/mL for AdsiRNA-survivin and 2.1×10^9 pfu/mL for AdU6-control. The HepG2 cell line was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Science (SIBCB) and kept at this lab. Four-week-old BALB/c nude mice were purchased from SIBCB; half were male and half were female.

2.2. *Construction of an animal model of hepatocarcinoma using nude mice*

The HepG2 cell line was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were incubated at 37°C with CO_2 saturation of 5%. Cells were harvested until 70-80% confluence was observed. They were then washed with PBS, suspended, and prepared in a 2×10^6/mL single-cell suspension. Mice were given a hypodermic injection of 2 mL suspension in the back and the growth of transplanted human hepatocarcinoma was observed.

2.3. *Animal experiments*

The recombinant adenovirus was used in 2 working doses: 2×10^9 pfu/mL and 2×10^8 pfu/mL. The control groups were an AdU6-control (2×10^9 pfu/mL) and a PBS control. The BALB/c nude mice were randomly divided into four groups when the tumor diameter reached 1 cm with 3 mice per group. These four groups were identified as the high survivin dose group, the low survivin dose group, the AdU6-control group, and the PBS control group. One hundred μL of adenovirus were injected straight into tumor tissue on days 1, 2, 10, 20, and 30, respectively, and mice were sacrificed 5 days after the injection was complete.

2.4. *Depiction of the tumor growth curve*

Tumor size was measured 2 days before injection and 5 days afterwards. Tumor volume was determined by the formula tumor length × tumor width^2 × 0.4 (tumor length, the tumor's longest diameter; tumor width, the shortest diameter perpendicular to the tumor length). A tumor growth curve was determined according to the tumor volume.

2.5. *Detection of DNA segments in hepatocarcinoma cells*

DNA segments in hepatocarcinoma cells were detected by TUNEL assay, which was performed in accordance with the in situ Cell Death Detection Kit (Roche Molecular Biochemicals) instruction manual.

2.6. *Statistical analysis*

The results of the tests were analyzed using SPSS10.0 software. The difference between groups was compared using ANOVA. A p value of less than 0.05 was considered to be significant.

3. Results

3.1. *Observation of AdsiRNA-survivin therapeutic effectiveness against transplanted human hepatocarcinoma in nude mice*

The growth of tumors in nude mice injected with HepG2 cells was inhibited with the greater duration of injection with AdsiRNA-survivin for both the high and low dose groups, but inhibition was more apparent in the high dose group. The tumor volume was larger in the PBS and AdU6-control groups. The tumor volume in the four groups was as follows: high dose of AdsiRNA-survivin > low dose of AdsiRNA-survivin > AdU6-control > PBS control (Figure 1). The tumor growth curve (Figure 2) depicted by the tumor volume indicated that the AdsiRNA-survivin adenovirus obviously inhibited transplanted human hepatocarcinoma in nude mice. This effect was directly proportionate to the injected dose and adenovirus concentration, but tumor growth in the AdU6-control group was similar to that in the PBS control group.

3.2. *Detection of apoptosis in animal tumor cells by TUNEL assay*

Results of the TUNEL assay are shown in Figure 3. Most nuclei in the mouse tumor cells infected with either a high or low dose of AdsiRNA-survivin were brown in color and had a high nucleo-cytoplasmic concentration and irregular cell shape. A great deal of apoptotic cells appeared in these two groups but few appeared in the AdU6-control and PBS control groups. The apoptotic cell count of the four groups was as follows: high dose of AdsiRNA-survivin > low dose of AdsiRNA-survivin > AdU6-control > PBS control.

Six fields of vision (400× high power filed, 100 cells per filed) under a high-powered microscope were selected to count the number of cells that were apoptosis-positive, and results were calculated as the mean ± standard deviation. Numbers of apoptotic human hepatocarcinoma cells are shown in Table 1. Results indicated that the number of apoptotic cells in the groups with a high or

Table 1. The number of apoptotic cells in three groups

Group	Apoptotic cell count
High dose of AdsiRNA-survivin	34.67 ± 2.70
Low dose of AdsiRNA-survivin	27.41 ± 2.42
AdU6-control	3.57 ± 1.61

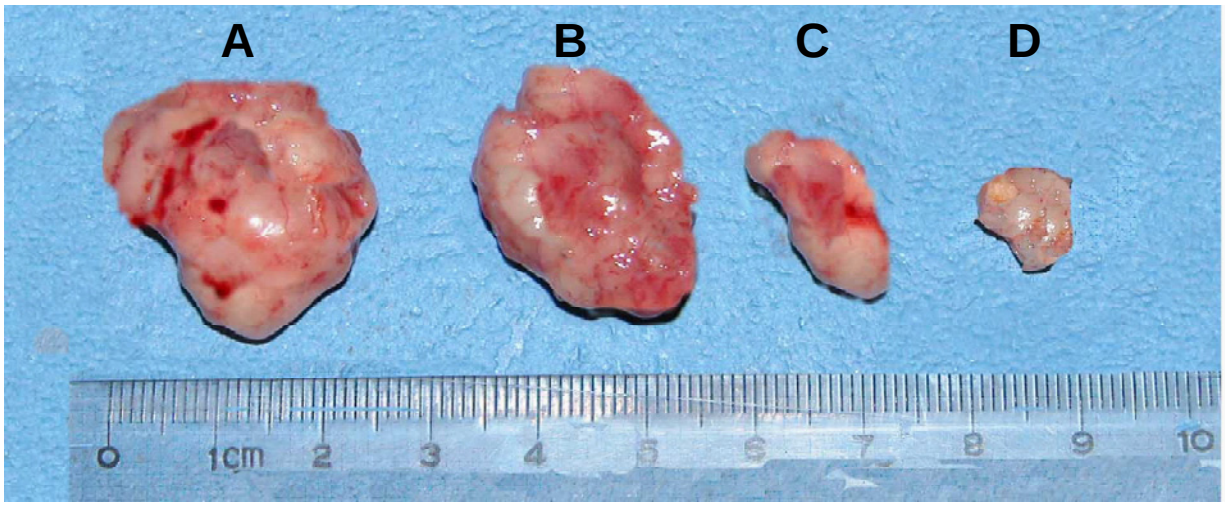

Figure 1. The size of tumors in mice after treatment with recombinant adenovirus-siRNA. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.

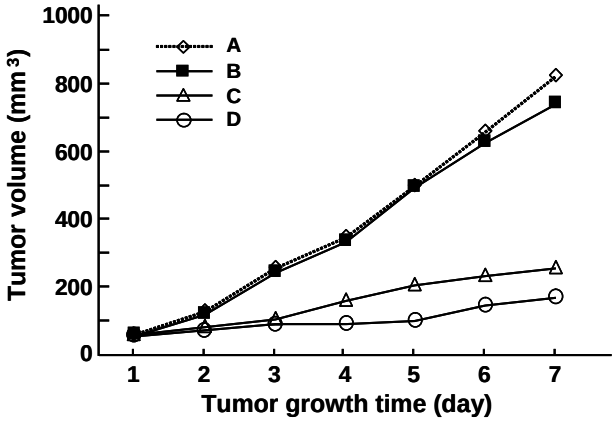

Figure 2. Growth curve of tumors. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.

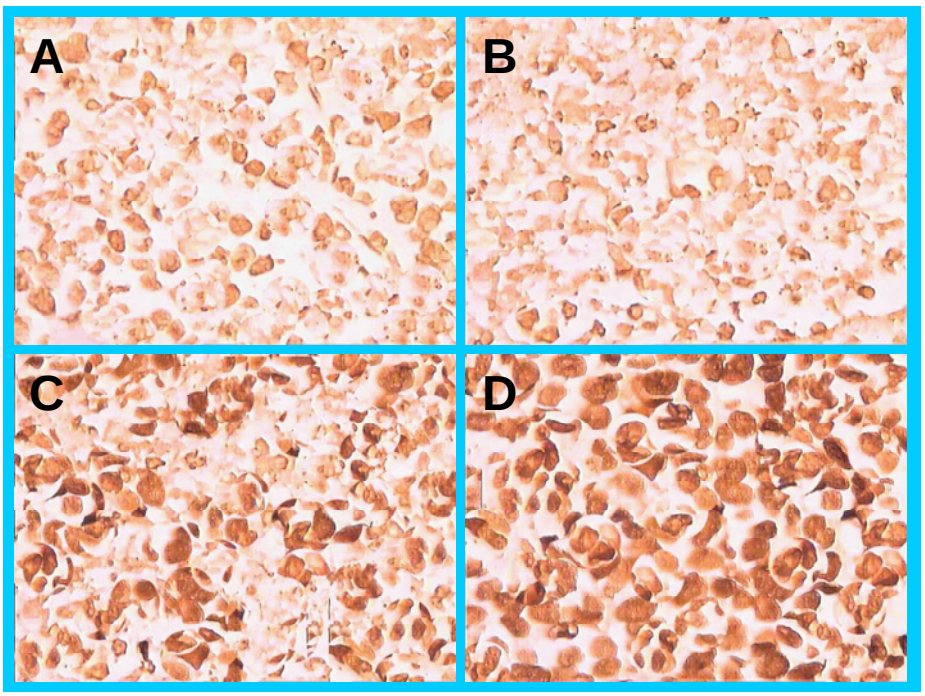

Figure 3. Detection of apoptotic cells in tumors by TUNEL after infection with AdsiRNA. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.

low dose of AdsiRNA-survivin differed significantly from that in the AdU6-control group ($p < 0.01$).

4. Discussion

In recent years, the new technology of RNAi has been used to inhibit target genes and research their effects and mechanism of action, but most RNAi experiments have used a plasmid-mediated siRNA vector. As the plasmid-mediated siRNA vector has some limitations such as its problematic use in animal trials and difficulty in demonstrating its effect on the target gene, a new RNAi system was established using adenovirus as the siRNA vector. In this study, the construction of the AdsiRNA-survivin was based on a plasmid-mediated siRNA-survivin vector harvested by cell infection *in vitro*. The AdsiRNA-survivin adenovirus is highly infective to various cell lines and host types and can effectively inhibit survivin expression in hepatocarcinoma cells *in vitro* (*2*). In the current study, it was used in an animal trial *in vivo* to prove that a viral-mediated siRNA vector could be easily used in animal experiments.

In this study, IAP survivin was selected as the target gene. Survivin obviously counteracted apoptosis in tumor cells. A feature readily distinguishing it from other IAPs is that it is not expressed in normal tissue (except for thymus gland) but is commonly expressed in human tumor cell lines. Tamm *et al*. (*3*) investigated the antiapoptotic mechanism of survivin and as its expression in 60 human tumor cell lines; they indicated that survivin was expressed in all 60 cancer cell lines analyzed, with the highest levels in breast and lung cancer and lowest levels in renal cancer. As demonstrated by immuno-histochemistry, Western blot, and in situ hybrid, survivin is prominently expressed *in vivo* in all the most common human cancers of the lung, colon, pancreas, prostate, and breast but is not detected in normal cells, suggesting that survivin is a potential new target for apoptosis-based therapy for cancer (*4*). Studies have reported that cells infected by various means such as plasmid siRNA, manual synthetic antisense oligonucleotides, antisense RNA, and negative dominance mutants displayed inhibited survivin expression and apoptosis (*5-7*). In the current study, an animal model of hepatocarcinoma using an immunodeficient mouse was created and adenovirus-mediated RNAi technology was used in a mammal; results demonstrated that the growth of the transplanted hepatocarcinoma was obviously inhibited and that apoptosis was induced in a number of cells by inhibiting survivin expression. These results represent credible trial experience with hepatocarcinoma gene therapy and establish a trial basis for subsequent research into survivin's inhibition mechanism and its role in the apoptosis signaling pathway.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (No. 30371599) and the Natural Science Foundation of Shandong Province, China (No.Y2007C152).

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(*Received November 16, 2008; Accepted December 14, 2008*)

Original Article

Multilevel analysis of solar radiation and cancer mortality using ecological data in Japan

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Summary **A preventive effect of solar radiation on cancer has been suspected. This study aimed to compare the statistical relationship between solar radiation and cancer mortality according to hierarchical models and adjustment for confounding factors, and then to demonstrate the relationship with main site-specific cancer mortalities in Japan. We examined the relationship between all-site and main site-specific cancer mortalities and global solar radiation using Poisson regression with municipal data around 2000. The models included single-level (municipality) and multilevel (municipality and prefecture) with/without potential confounding factors (lifestyle and socioeconomic variables). For all-site cancer, single-level analysis showed a significant, strong negative association with solar radiation. However, multilevel analysis showed a moderate or no association. In multilevel analysis with potential confounding factors, solar radiation was significantly negatively associated with most site-specific cancers, but not with gallbladder and liver cancer in men and stomach and breast cancer in women. Our findings support the preventive effective of solar radiation on several types of cancer. However, to show a concrete relationship, a statistical model with an appropriate hierarchy and adjustment for potential confounding factors is required.**

Keywords: Malignant neoplasm, Solar radiation, Vitamin D, Multilevel analysis, Ecological study

1. Introduction

The influence of solar radiation on cancer has recently received attention (*1*). Some epidemiological studies have suggested a preventive effect of solar radiation on several types of cancer, such as colonic (*2-4*), breast (*5,6*), lung (*7,8*), pancreatic (*9,10*), prostatic (*11*), and ovarian cancer (*12*). In addition to epidemiological studies in western countries, a few studies in Asian countries including Japan (*13-15*) support the protective effect of solar radiation against cancer.

Investigation of the relation between solar radiation and cancer mortality predominantly depends on ecological studies, since individual-level exposure to solar radiation is difficult to measure (*16,17*). Ecological studies, however, have several critical weaknesses in providing causal evidence, including confounding and ecological fallacy (*18*). Waltz and Chodick suggested that the association of solar radiation with cancer mortality resulted from confounding effects caused by ecological study design (*19*). In Japan, prefectural-level analysis has a small unit size (n = 47), and thus, possible confounders might not be sufficiently considered. Municipal-level analysis with a large number of units (about 2,000) can deal with several possible confounders, although some kinds of important data such as life-style related variables are not available. There has been little discussion to compare results among different study designs and to elucidate which study design is suitable to detect the true relationship between solar radiation and cancer mortality.

Multilevel analysis has been used for various public

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health and epidemiological studies (*20*,*21*). It detects the influence of hierarchical levels (*e.g.*, individual and neighborhood levels) and their interaction. Multilevel analysis can also be applied to not only individual data but also ecological data (*20*,*22*,*23*). However, it is not clear whether multi-level analysis demonstrates different and more valid evidence than single-level analysis.

This study compared the results of different types of ecological studies, including single-level and multilevel models with and without adjustment for possible confounding variables, on the relationship between solar radiation and cancer mortality in Japan. In addition, its relationship with major sites of cancer was examined using multilevel ecological analysis.

2. Methods

2.1. *Data*

The unit of analysis was basically municipalities: cities, towns, villages, and wards ("ku") of several designated cities. In 2002, there were 3,000 municipalities in Japan. The local governments of Japan have two hierarchical systems: prefectures and municipalities. There are 47 prefectures, which consist of a few tens of municipalities.

Mortality was based on deaths from 1998 to 2002 (*24*). The expected number of deaths in municipalities was estimated using the sex-age (five-year interval)-specific population and the national mortality rate in 2002. We estimated empirical Bayes estimates of local standardized mortality ratio (EBSMR) from all-site cancer of municipalities using maximum likelihood method of Poisson-Gamma model with the secondary medical care zones as groups of municipalities to estimate empirical prior distribution (*22*,*23*,*25*). The estimation of EBSMR was conducted by a window based program developed by Nakaya (*26*).

The data on solar radiation for this study were constructed as population-weighted mean annual global solar radiation of municipalities (MJ/m^2). According to the Standard Grid Square (*27*), the population as of 2000 based on the national population census and climate summary statistics including annual mean solar radiation during the period of 1971-2000 were compiled as grid square statistics based on a small square unit defined as 30" latitude × 45" longitude (approximately 1 square kilometer). Overlaying these gridded data with a municipality zoning layer, we calculated the population-weighted average value of global solar radiation for each municipality in a GIS environment. The original gridded data of 30-year mean climate summaries are provided as Mesh Climatic Data 2000 (*28*) in which the amount of global solar radiation was computed by the gridded duration of sunshine, which was interpolated by applying a multiple regression technique to the records of meteorological stations with elevation and urban indices. It should also be noted that adjustment for the effect of elevation, that is, shadowing of land features, was made for computing the gridded data of global solar radiation.

We used socioeconomic and lifestyle data as potential confounding factors. Since municipality-level data of dietary and nutritional intake were not available, we used prefecture-level data of these variables (*29*). Using principle component analysis with eighteen items of dietary consumption (*e.g.*, rice, potato, beans, fruit and green vegetables, and egg) (*30*), we drew five components and thus we used the factor scores of these components. An additional lifestyle variable was smoking rate, which was obtained from the Comprehensive Survey of the Living Conditions of People on Health and Welfare ("Kokuminseikatu Kiso Chosa") in 2004 conducted by the Japanese Ministry of Health, Labour and Welfare (*31*). The socioeconomic variables consisted of per capita income, unemployment rate, and population density, which were municipality-level data. Previous studies have demonstrated a strong relationship between these factors and all-cause and main leading causes of death in Japan (*22*,*23*).

2.2. *Analysis*

The relationship between mortality and solar radiation was evaluated by Poisson regression analysis, which is described in previous studies (*22*,*23*). We used the following six models: Model 1 was single-level (municipality) analysis without adjustment; Model 2 was single-level analysis with adjustment for socioeconomic variables; Model 3 was single-level analysis with adjustment for socioeconomic and municipal variables; Model 4 was multilevel (municipality and prefecture) analysis without adjustment; Model 5 was multilevel analysis with adjustment for socioeconomic variables; and Model 6 was multilevel analysis with adjustment for prefectural and municipal data.

The equation for Poisson regression was as follows; O_{ij} is the observed death number, E_{ij} expected death number, x_{nj} a variable of a potential confounder of j prefecture, x_{nij} that of i municipality in j prefecture, and u is a random effect among prefectures.

Single-level:

$$\log(O_{ij}) = \log(E_{ij}) + \beta_0 + \beta_{1j}x_{1j} + \ldots + \beta_{nj}x_j + \beta_{1i}x_{1ij} + \ldots + \beta_{ni}x_{nij}$$

Multilevel:

$$\log(O_{ij}) = \log(E_{ij}) + \beta_0 + u_j + \beta_{1j}x_{1j} + \ldots + \beta_{nj}x_{ij} + \beta_{1i}x_{1ij} + \ldots + \beta_{ni}x_{nij}$$

We used SPSS 15.0J (Chicago, SPSS Inc.) for principle component analysis and MLwiN 2.0 (London,

Centre for Mutilevel Modelling, Institute of Education, University of London) for Poisson regression analysis.

3. Results

Figure 1 is a map showing all-site cancer mortality (EBSMR) and global solar radiation of municipalities. The EBSMR and the global solar radiation ranged from 0.44 to 1.48 and from 11.1 to 15.8 (MJ/m^2), respectively. The southern part and mountainous areas had higher solar radiation. In contrast, the northeast part showed a lower level of solar radiation.

Figure 2 shows the results of Poisson regression of the relation between all-cause mortality and solar radiation. Model 1 to Model 3 are single-level models with a single regression line, while Model 4 to Model 6 are multilevel models with prefectural specific regression lines (n = 47). Single-level analysis (Models 1, 2 and 3) showed a significant relationship regardless of adjustment for potential confounding factors. However, Model 4 of multilevel analysis did not show a significant relationship. When the municipal data (socioeconomic variables) were adjusted for (Model 6), mortality and solar radiation showed a significant negative association.

Table 1 shows the results of analysis of the relationship between solar radiation and male cancer mortality using three models of Poisson regression. In Model 1, a significant negative relationship was found for all cancer mortalities except for liver cancer. Model 4 showed a significant negative relationship for esophageal and pancreatic cancer, and a significant

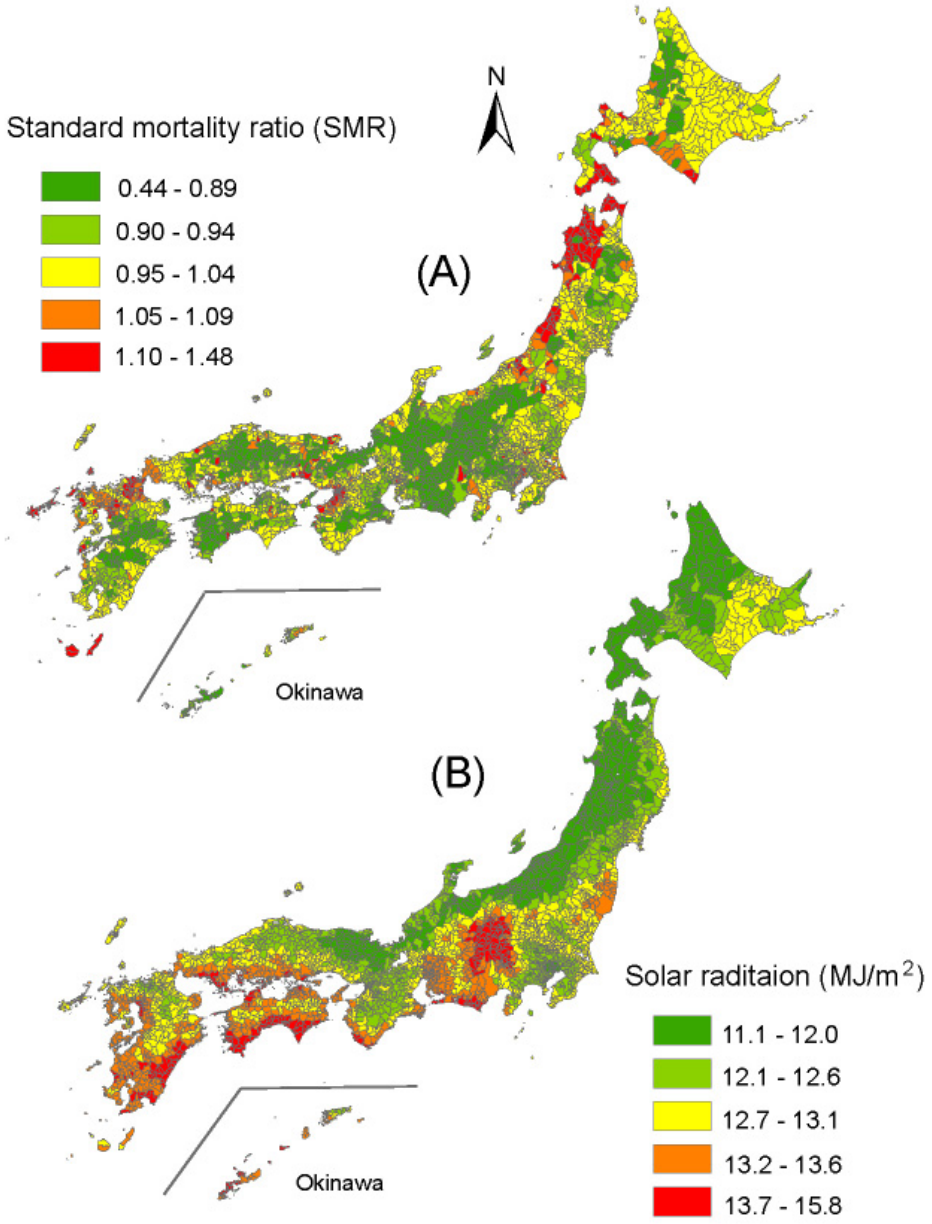

Figure 1. Mapping of mortality empirical Bayes estimates of SMR of all-site cancer (A) and global solar radiation (B) by municipality in Japan.

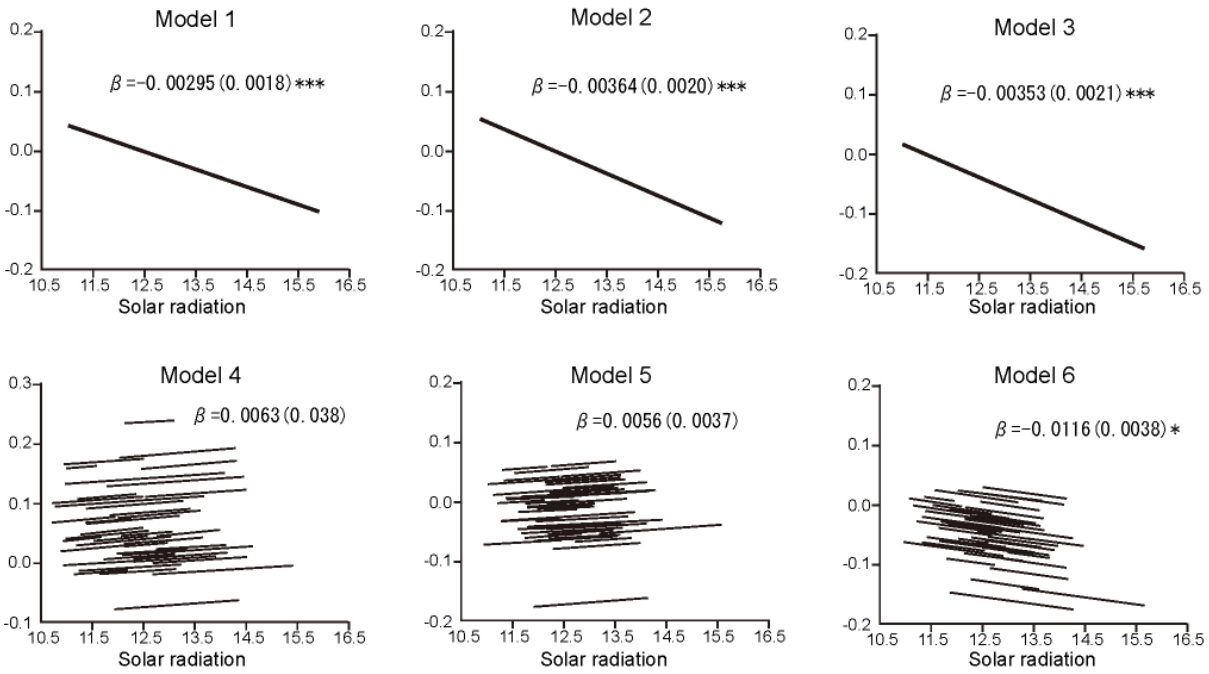

Figure 2. Relationship between solar radiation and all-site cancer mortality for men. The x axis is solar radiation (MJ/m^2) and the y axis is log RR (= SMR) predicted by Poisson regression. Model 1: single-level without adjustment; Model 2: single-level with adjustment for prefectural data; Model 3: single-level without adjustment for prefectural and municipal data; Model 4: multilevel without adjustment; Model 5: multilevel with adjustment for prefectural data; Model 6: multilevel with adjustment for prefectural and municipal data. * $p < 0.05$, *** $p < 0.001$.

positive relationship for stomach and liver cancer. In the final model (Model 6), a significant negative relationship with solar radiation was found for all cancers except for gallbladder and liver cancers.

Table 2 shows the results for female cancer. In single-level analysis (Model 1 and Model 2), all mortalities were significantly negatively associated with solar radiation. In Model 4, colorectal, gallbladder, and pancreatic cancers showed a significant negative relationship. In addition to these three cancers, all-site cancer and lung cancer showed a significant negative relationship with solar radiation.

4. Discussion

This study demonstrated that the statistical relationship between solar radiation and all cancer mortality differed among statistical models. For male all-site cancer, a single-level model showed a significant negative relationship regardless adjustment for potential confounders. In a multilevel model, however, this relationship was not found. This difference suggests that the relationship in the single-level model is not true, since the true relationship should be independent of the hierarchical model. The relationship in the single-level (municipal level) model might be confounded by unknown and unavailable factors of prefectural level variables. It is suggested that the negative association between solar radiation and cancer mortality in a previous study using single (prefecture) level analysis (*15*) might be influenced by confounding factors and fallacies.

The relationship between all-site cancer mortality and solar radiation was similar among models with and without lifestyle-related variables. This similarity was also found in most site-specific cancer mortality. It is suggested that these variables, which were used in a previous study (*15*), are not useful as variables for adjustment. In contrast, adjustment for socioeconomic variables (municipal level) greatly modified the relationship between mortality and solar radiation. These socioeconomic variables are useful potential confounders, although it might be dummy variables including unknown and unavailable factors. Even in the final model adjusting lifestyle and socioeconomic variables, there was large variation among prefectures, as shown in Figure 2. This suggests that the variation might depend on other unknown factors, such as medical resources.

One study in Japan investigating pancreatic cancer mortality and solar radiation did not consider any kind of factors related to lifestyle (*14*). Even if the study had shown a negative association between solar radiation and mortality, the result would seem incredible. Another Japanese study considered some possible confounding factors related to lifestyle (*15*). The adjustment, however, hardly changed the relationship, and thus it might not have included important confounding factors.

Concerning methodological issues, our analysis has three advantages compared with previous studies. First, the use of multilevel analysis could adjust for unknown and measurable confounding factors. Second, we used

Table 1. Results of Poisson regression analyses for solar radiation and cancer mortality in Japan according to various models: men

Site of cancer	Model 1		Model 2		Model 4		Model 6	
	Coefficient	(SE)	Coefficient	(SE)	Coefficient	(SE)	Coefficient	(SE)
All sites	-0.0295	(0.0018)***	-0.0364	(0.0020)***	0.0067	(0.0038)	-0.0116	(0.0038) **
Esophagus	-0.2226	(0.0082)***	-0.1606	(0.0093) ***	-0.1099	(0.0173) ***	-0.1160	(0.0175) ***
Stomach	-0.1546	(0.0040)***	-0.0723	(0.0046) ***	0.0259	(0.0087) **	-0.0255	(0.0089) **
Colorectum	-0.0885	(0.0053)***	-0.0625	(0.0060) ***	-0.0189	(0.0103)	-0.0267	(0.0100) **
Gallbladder	-0.0149	(0.0089)	-0.0079	(0.0099)	-0.0160	(0.0161)	-0.0180	(0.0133)
Pancreas	-0.0686	(0.0073) ***	-0.0617	(0.0083) ***	-0.0410	(0.0130) **	-0.0357	(0.0113) **
Liver	0.1209	(0.0048) ***	0.0656	(0.0055) ***	0.0637	(0.0107)***	0.0293	(0.0110) **
Lung	-0.0183	(0.0037) ***	-0.0507	(0.0043) ***	-0.0024	(0.0077)	-0.0299	(0.0076) ***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Model 1: Single-level (prefecture) without adjustment
Model 2: Single-level (prefecture) with adjustment for dietary factors and smoking rate
Model 4: Multi-level (prefecture and municipality) without adjustment
Model 6: Multi-level (prefecture and municipality) with adjustment for dietary factors, smoking rate and socioeconomic conditions

Table 2. Results of Poisson regression analyses for solar radiation and cancer mortality in Japan according to various models: women

Site of cancer	Model 1		Model 2		Model 4		Model 6	
	Coefficient	(SE)	Coefficient	(SE)	Coefficient	(SE)	Coefficient	(SE)
All sites	-0.0474	(0.0022)***	-0.0369	(0.0026)***	-0.0021	(0.0045)	-0.0146	(0.0044)***
Stomach	-0.0745	(0.0055)***	-0.0644	(0.0065)***	-0.0052	(0.0115)	-0.0121	(0.0118)
Colorectum	-0.1035	(0.0058)***	-0.0724	(0.0069)***	-0.0529	(0.0108)***	-0.0516	(0.0110)***
Gallbladder	-0.0359	(0.0080)***	-0.0244	(0.0094)**	-0.0338	(0.0146)*	-0.0373	(0.0138)**
Pancreas	-0.0736	(0.0078)***	-0.0478	(0.0094)***	-0.0494	(0.0128)***	-0.0316	(0.0118)**
Breast	-0.1014	(0.0079)***	-0.0255	(0.0096)**	-0.0089	(0.0155)	0.0165	(0.0131)
Lung	-0.0384	(0.0060)***	-0.0382	(0.0073)***	0.0069	(0.0124)	-0.0226	(0.0112)*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Model 1: Single-level (prefecture) without adjustment
Model 2: Single-level (prefecture) with adjustment for dietary factors and smoking rate
Model 4: Multi-level (prefecture and municipality) without adjustment
Model 6: Multi-level (prefecture and municipality) with adjustment for dietary factors, smoking rate and socioeconomic conditions

potential confounding factors as much as possible. Last, Poisson regression analysis could compare not the correlation coefficient, but the regression coefficient. Because of these three advantages, our analysis approached the true relationship between solar radiation and cancer mortality.

Using the final model, multilevel analysis with adjustment for socioeconomic and dietary variables, we examined the relationship between solar radiation and cancer mortality of main sites. Solar radiation was significantly negatively associated with most gastrointestinal cancers and male lung cancer. These findings agree with previous studies, which showed a beneficial effect of solar radiation on these cancers (*1*).

The beneficial effect of solar radiation on cancer is partly explained by vitamin D. Epidemiological studies including a cohort study and intervention study demonstrated evidence that high serum levels of vitamin D are associated with lower risk of some cancers (*1*). The evidence suggested that the beneficial effects of sunlight against cancer might be mediated by its role in vitamin D production.

This study found an inverse effect of solar radiation on liver cancer. Since it is not reasonable that solar radiation increases the risk of liver cancer, even the final model seems to suffer from remaining confounding factors. For female breast cancer, on which solar radiation has been demonstrated to have a beneficial effect in western countries (*5,6*), the final model of this study showed no relationship with solar radiation. This inconsistency suggests that differences in the incidence/mortality and the strength of other risk factors among countries could contribute to the impact of solar radiation.

In conclusion, this study attempted to examine the suitability of different statistical models in relation to solar radiation and cancer mortality, and demonstrated that multilevel analysis with adjustment for relevant possible confounding factors is more suitable than single-level analysis. Using multi-level analysis, our findings support the preventive effective of solar radiation on several types of cancer, especially gastrointestinal cancer.

Acknowledgement

This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare (H18-Ganrinsho-Ippan-016).

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(*Received November 17, 2008; Accepted Dcember 7, 2008*)

Original Article

Influence of selective brain cooling on the expression of ICAM-1 mRNA and infiltration of PMNLs and monocytes/macrophages in rats suffering from global brain ischemia/reperfusion injury

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Summary **This study sought to evaluate the effects of selective brain cooling on the expression of intercellular adhesion molecule-1 (ICAM-1) mRNA and infiltration of polymorphonuclear leukocytes (PMNLs) and monocytes/macrophages (M*Φ*) during global cerebral ischemia/reperfusion (I/R). Global ischemia of the brain was produced by four-vessel occlusion for 30 min followed by reperfusion for 240 min. Thirty-five SD rats were randomly divided into five groups: group I had no ischemia and reperfusion; groups II, III, IV, and V were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 37, 35, 32, and 28°C, respectively. Cerebral tissue samples were taken for pathological examination of the infiltration of PMNLs and M*Φ* and to detect ICAM-1 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR). The expression of ICAM-1 mRNA and infiltration of PMNLs and M*Φ* increased more markedly in group II than in group I ($p < 0.01$), suggesting that hypothermia evidently inhibited ICAM-1 mRNA expression and PMNL and M*Φ* infiltration in the damaged cerebral tissue. In addition, significant differences were also found between group III and group II ($p < 0.05$) and among groups IV, V, and II ($p < 0.01$). These results suggest that I/R injury induces ICAM-1 mRNA expression and PMNL and M*Φ* infiltration in SD rats and that selective brain cooling, and especially moderate hypothermia (28-32°C), may provide better cerebral protection by markedly inhibiting the expression of ICAM-1 mRNA while decreasing the infiltration of PMNLs and M*Φ* in the brain.**

Keywords: Hypothermia, Ischemia/reperfusion, Intercellular adhesion molecule-1 mRNA, Polymorphonuclear leukocytes, Monocytes/macrophages

1. Introduction

Activation and infiltration of leucocytes, and especially polymorphonuclear leukocytes (PMNLs) and monocytes/macrophages (M*Φ*), is a major factor that results in ischemia/reperfusion (I/R) injury after brain ischemia (*1*). Recent evidence has revealed the crucial role cell-adhesion molecules play in inflammation-induced rolling, adhesion, and accumulation of PMNLs and M*Φ* in tissues (*2*). An important adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) plays a key role in PMNL and M*Φ* adhesion to endothelium and migration into injured tissues (*3*).

Inhibiting the expression of ICAM-1 and blocking the infiltration of PMNLs and M*Φ* have been shown to decrease I/R injury (*4*). Hypothermia provides brain protection by decreasing oxygen consumption, reducing cerebral edema, and inhibiting excitatory amino acid and oxyradical generation (*5*). However, the mechanism of brain protection has not been fully elucidated. The current study investigated whether hypothermia, and especially moderate hypothermia (28-32°C), affects modulation of ICAM-1 expression and PMNL and M*Φ* infiltration in injured cerebral tissue.

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2. Material and Methods

2.1. *Animals*

Sprague-Dawley rats (weight, 180-200 g; n = 35) were used in the experiments. All procedures were approved by the Animal Care and Use Committee of Nanjing University and were in accordance with the NIH guidelines for the ethical use of laboratory animals. Animals were deprived of food for 8 h before surgery and given free access to water. All animals were anesthetized with ketamine (80 mg/kg, i.p.).

2.2. *Four-vessel occlusion model*

A four-vessel occlusion model was used to induce global cerebral ischemia/reperfusion (*6,7*). Under ketamine anesthesia, a midline incision (1 cm in length) was made in the dorsal neck, the paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed. As described by Green (*8*), the rat's vertebral arteries travel within the vertebral canal and pass beneath the alar foramen before entering into the posterior fossa. A pin 0.5 mm in diameter was inserted through each alar foramen and both vertebral arteries were cauterized and permanently occluded. The foramina were packed with bone wax, and the muscles and fascia were closed in layers. Through a ventral midcervical incision, each carotid artery was isolated and a 9-0 nylon ligature was looped around it. After 24 h, cerebral ischemia was induced by traction on both carotid ligatures; then, bilateral ligatures were loosened and reperfusion was performed for 4 h. The rats were sacrificed and cerebral tissue was quickly removed. Some cortical samples were immediately frozen in liquid nitrogen and stored at –80°C and other samples were fixed with formaldehyde.

2.3. *Temperature control*

Since some studies have indicated that there was no significant difference between inner ear and brain temperatures (*9*), inner ear temperature was monitored instead of brain temperature in the present experiment. Animals were cooled with ice packs.

2.4. *Experimental grouping*

Animals were randomly divided into the following five groups (Table 1): group I (n = 7) animals had no ischemia or reperfusion, group II animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 37°C, group III animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 35°C, group IV animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 32°C, and group V animals (n = 7) were subjected to ischemia for 30 min at 37°C and reperfusion for 240 min at 28°C.

Table 1. I/R conditions in animal groups

	Animal groups				
	I	II	III	IV	V
Ischemic time (min)	0	30	30	30	30
Reperfusion time (min)	0	240	240	240	240
Temperature of reperfusion (°C)	37	37	35	32	28
Number of animals	7	7	7	7	7

2.5. *Determination of ICAM-1 mRNA level by RT-PCR*

Total cellular RNA was extracted from cortical samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The following primers were used in this study: ICAM-1 cDNA (amplification product 590 bp) — sense primer: 5'-AAGGTGTGATATCCGGTAGA-3', antisense primer: 5'-CCTTCTAAGTGGTTGGAACA-3'; β-actin cDNA (amplification product 348 bp) — sense primer: 5'-TA AAGACCTCTATGCCAACAC-3', antisense primer: 5'-TAAAGCCATGCCAAATGTCTC-3'. β-actin was used as an internal control in PCR. ICAM-1 mRNA was reverse-transcripted at 48°C for 45 min. Amplification was performed according to the following conditions: samples were initially denatured at 94°C for 2 min followed by 30 cycles of amplification (30-sec denaturing at 94°C, 1-min annealing at 60°C, and 2-min extension at 68°C) and a final 7-min extension at 68°C. The PCR product was electrophoresed through agarose gel. The gel was dried and autoradiographed. The PCR-amplified DNA bands of ICAM-1 and β-actin were quantitated by phosphorImager analysis. The ratios of ICAM-1 to β-actin were calculated from the co-amplified samples, and relative levels of ICAM-1 mRNA were determined based on the differences of these ratios.

2.6. *Histopathology*

Brain tissues were processed and embedded in paraffin, and 4-μm-thick paraffin sections were stained with hematoxylin-and-eosin for histopathological evaluation. Six random high-double views (magnification, ×100) were taken to count PMNLs and M*Φ*.

2.7. *Statistical analysis*

All values are present as mean ± standard error. Statistical evaluation was performed using ANOVA followed by a Q test. A significant difference was indicated by $p < 0.05$, and a highly significant difference was indicated by $p < 0.01$.

3. Results

3.1. *Infiltration of PMNLs and MΦ*

Table 2 summarizes the absolute values of tissue

PMNL and MΦ counts for the various groups. Group II exhibited significantly more PMNLs and MΦ in cortical tissues than Group I ($p < 0.01$). In comparison to Group II, the counts of PMNLs and MΦ decreased significantly in other groups ($p < 0.05$, $p < 0.01$).

3.2. *Expression of ICAM-1 mRNA in various groups*

Expression of ICAM-1 mRNA in cerebral tissue was detected by RT-PCR. The quantitative data for ICAM-1 mRNA, after normalization to β-actin in each sample, were summarized. As shown in Figure 1, only a low basal level expression of the ICAM-1 mRNA was detected in the non-ischemic rat (group I). The expression of the 590 bp ICAM-1 mRNA was induced by I/R injury (Figure 1, group II). Figure 2 shows the RT-PCR of ICAM-1 mRNA expression at various temperatures. In comparison to group II, significant downregulation of ICAM-1 mRNA was noted in hypothermic groups ($p < 0.05$, $p < 0.01$) (Table 3).

4. Discussion

Recent evidence has indicated that reperfusion itself may be detrimental to the ischemic tissue and that leucocytes, and especially PMNLs and MΦ, play an important role in the development of ischemia-reperfusion injury (*10*). One of the proposed mechanisms is "no-reflow phenomenon"-microvascular occlusion caused by accumulated leucocytes, mechanical obstruction, and vasoconstrictive mediator release (*11*). In addition to acute microvascular occlusion, leukocytes may also facilitate ischemic injury by enhancing the blood-brain barrier, infiltrating ischemic tissue, or initiating thrombosis (*12*). They may also lead to parenchyma injury *via* protein hydrolytic enzyme release, lipid mediator production, or oxygen radical production due to activated inflammatory cell infiltration and the respiratory burst (*13*). Depletion of leucocytes has been found to alleviate brain injury after I/R (*14*). The current results indicate that the number of PMNLs and MΦ closely correlates with the extent of cerebral tissue damage.

The current view is that adhesion of leukocytes to microvascular endothelium is a critical stage in the migration of leukocytes into injured tissues (*15*). The adhesion is regulated in part by ICAM-1 in endothelial cells and a group of CD11/CD18 glycoproteins in leukocytes (*16*). An increase in leukocyte adherence to endothelial cells has been reported in ischemic/reperfused tissue, and interactions between ICAM-1 and CD11/CD18 may be involved in this process (*16*).

A lack of sensitivity to I/R injury has been noted in mice with the ICAM-1 and CD11a/CD18 genes knocked out (*17*). Pretreatment with anti-ICAM-1 antibodies and anti-CD11a/CD18 antibodies significantly reduces cerebral ischemic cell damage by blocking leukocyte endothelial adhesion and migration. A deficiency in ICAM-1 attenuates microcirculatory disturbance and infarction size in focal cerebral ischemia (*18,19*).

In international, hypothermia was plotted out mild hypothermia (33-35°C), moderate hypothermia

Table 2. PMNL and MΦ infiltration in cerebral tissue in various groups

Groups	Infiltration [a,b]	
	PMNLs	MΦ
I	2.1 ± 1.5**	0.2 ± 0.1**
II	10.3 ± 1.1	2.0 ± 0.3
III	8.4 ± 2.4*	1.6 ± 0.6*
IV	6.5 ± 0.7**	1.3 ± 0.4**
V	6.1 ± 0.8**	1.2 ± 0.3**

[a] Data are represented as the mean ± SE; [b] In comparison to group II: * $p < 0.05$, ** $p < 0.01$.

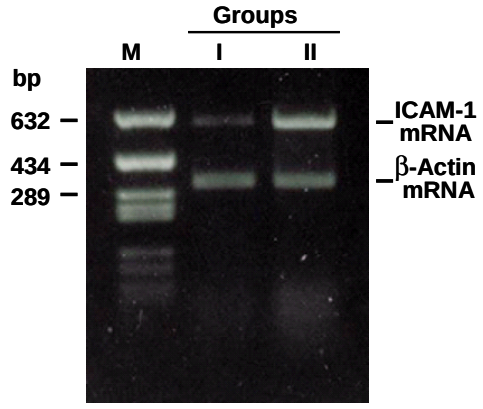

Figure 1. Expression of ICAM-1 mRNA in I/R injury. β-Actin mRNA was used as an internal control in PCR. Lane M, PCR marker.

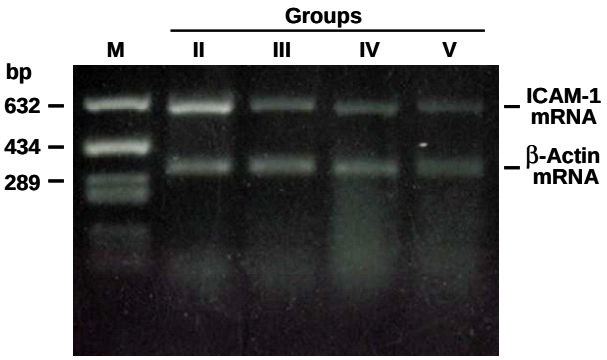

Figure 2. Expression of ICAM-1mRNA at various temperatures. β-Actin mRNA was used as an internal control in PCR. Lane M, PCR marker.

Table 3. ICAM-1 mRNA levels after normalization to β-actin in each sample

Groups	ICAM-1/β-actin mRNA [a,b]
I	0.264 ± 0.073**
II	1.825 ± 0.265
III	0.872 ± 0.151*
IV	0.447 ± 0.065**
V	0.433 ± 0.059**

[a] Data are represented as the mean ± SE; [b] In comparison to group II: * $p < 0.05$, ** $p < 0.01$.

(28-32°C), deep hypothermia (17-27°C) and super deep hypothermia (2-16°C) (*3*). Mild and moderate hypothermia offer acceptable brain protection. Possible contributing factors include i) decrease in oxygen consumption, a reduction in cerebral edema, inhibited excitatory amino acids, acetylcholine, dopamine, norepinephrine, 5-hydroxytryptamine, NO, and oxyradicals, ii) blockage of Ca^{2+} overload, iii) a decrease in the destruction or a replenishment of structural proteins of cerebral cells, and iv) alleviation of diffuse axial injury (*20*). Research has yet to be done on the effect of hypothermia on the expression of adhesion molecules or infiltration of PMNLs and M*Φ*. The current study found up-regulation of ICAM-1 mRNA expression and significant infiltration of PMNLs and M*Φ* after I/R injury. Hypothermia, and especially moderate hypothermia (28-32°C), could significantly inhibit the expression of ICAM-1 and infiltration of PMNLs and M*Φ*. These data suggest an optimal temperature (28-32°C) for the treatment of cerebral ischemia/reperfusion injury.

The mechanism by which ICAM-1 upregulation acts on cerebral endothelium during I/R injury is unclear. ICAM-1 is upregulated in the human brain and in microvascular endothelial cells by proinflammatory cytokines, tumor necrosis factor-α, and interleukin-1 (*21*). The mechanism of oxidation-reduction plays a role in modulating ICAM-1 expression (*22*). Another possibility is that the endothelium itself upregulates ICAM-1 in response to the stimulus of ischemia-reperfusion. The current study discovered a new mechanism of hypothermic brain protection *via* inhibition of ICAM-1 expression and blocking of PMNL and M*Φ* infiltration in I/R injured tissue. The issue of whether this hypothermic mechanism inhibits ICAM-1 expression directly or indirectly is unclear.

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(*Received December 12, 2008; Revised December 20, 2008; Accepted December 20, 2008*)

Original Article

Construction of an adenovirus vector carrying the human tissue inhibitor of metalloproteinase 2 gene

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Summary **This study sought to construct an adenoviral vector carrying the human tissue inhibitor of the metalloproteinase 2 (TIMP-2) gene for use in gene therapy. A recombinant adenovirus (AdTIMP-2) containing a human TIMP-2 cDNA fragment was generated by homologous recombination in BJ5183 bacteria. Recombinant plasmids were screened by antibiotic selection. The adenovirus vector was then packaged and amplified in HEK293 cells. A recombinant adenoviral vector carrying human TIMP-2 was constructed. The titer was 4×10^{11} pfu/mL after purification. The expression of the TIMP-2 gene in HEK293 cells was detected by PCR. A recombinant adenoviral vector carrying human TIMP-2 was successfully constructed and is available for further use in gene therapy for vascular disease.**

Keywords: Tissue inhibitor of metalloproteinase, Adenoviral vector, Gene therapy

1. Introduction

Abdominal aortic aneurysm (AAA) is a complex multifactorial disease, and inflammation appears to play a fundamental role in AAA development and progression (*1*,*2*). In a previous study, the current authors found that expression of matrix metalloproteinases (MMPs) increased in experimental abdominal aortic aneurysms in rats and that tetracycline inhibited the development of experimental abdominal aortic aneurysms *in vivo* through the inhibition of MMP-2 and MMP-9 expression (*3*,*4*). Many investigators have described the involvement of various members of MMP family in the degradation of extracellular matrix, and MMP-2 and MMP-9 in particular seem to play a pivotal role in this process (*5*,*6*). In addition, MMPs activity is regulated by the binding of endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs), 4 of which are now known. TIMP-2 selectively binds to pro-MMP-2, an interaction that can prepare the enzyme for activation by membrane type MMP-1 (MT1-MMP) (*7*). Therefore, the current study investigated the role of TIMP-2 in the formation of abdominal aneurysms using a recombinant adenovirus gene transfer system. An adenovirus vector carrying human TIMP-2 gene was first constructed and then the vector was propagated in human embryo kidney 293 (HEK 293) cells.

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2. Materials and Methods

2.1. *Construction of a recombinant adenovirus vector containing the human TIMP-2 gene*

A first generation (E1-, E3-) recombinant adenoviral vector was used to construct an expression vector for human TIMP-2 using the AdEasy™ system (Agilent Technologies, Inc., Santa Clara, CA, USA). This simplified system was first introduced by He TC, *et al.* (*8*). Briefly, the human TIMP-2 gene was released by *Eco*RI and *Xba* I digestion from the pGEM-4 vector

and inserted into the multicloning site (MCS) of the pBluescript vector, generating plasmid pBluescript-TIMP-2. The resultant plasmid was linearized by digestion with *Sal* I and *Xba* I. Then, the human TIMP-2 cDNA was inserted into the *Sal* I and *Xba* I restriction sites of the shuttle vector pAdTrack-CMV (cytomegalovirus). The resultant plasmid was designated the pAdTrack-CMV-TIMP-2 vector.

The resultant plasmid was linearized by digestion with *Pme* I and subsequently co-transformed into electrocompetent *E. coli* BJ5183 with an adenoviral backbone plasmid (pAdEasy-1; Agilent Technologies, Inc.). Electroporation was performed in 2.0 mm cuvettes at 2,500 V, 200 ohms, and 25 μF in a Gene Pulser electroporator (Bio-Rad Laboratories, Hercules, CA, USA). The cells were immediately placed in 500 μL of L-Broth and grown at 37°C for 20 min. One hundred twenty-five microliters of the cell suspension were then inoculated into four 10-cm Petri dishes containing L-agar plus 25 μg/mL of kanamycin. After 16-20 h of growth at 37°C, about 10-25 colonies per dish were obtained. Recombinants were selected for kanamycin resistance and were confirmed by restriction digestion with *Pac* I. Once recombinants were confirmed, supercoiled plasmid DNA was transformed into DH10B cells for large-scale amplification by electroporation.

Finally, the recombinant adenoviral plasmid was digested with *Pac* I and transfected into HEK 293 cells using the Lipofectamine (Invitrogen, Carlsbad, CA, USA) method for adenovirus packaging. Transfected cells were monitored for enhanced green fluorescence protein (GFP) expression and collected 7-10 days after transfection. The cells were lysed by three cycles of freezing and thawing. The viral lysates were then collected after centrifugation. The primary recombinant adenovirus with the human TIMP-2 (AdTIMP-2) was propagated by re-infecting HEK 293 cells and was purified by CsCl density gradient ultracentrifugation. Purified viruses were stored in phosphate-buffered saline (PBS) containing 10% glycerol at −80°C at a concentration of 2×10^{10} plaque-forming units (pfu)/mL. The control recombinant adenovirus AdCMV-GFP was similarly constructed.

2.2. *Identification of recombinant adenoviral particles by PCR*

On day 10, HEK 293 cells were collected and pelleted together along with any cells floating in the culture. After three cycles of freezing/thawing, 5 μL of viral lysate were used for detection of the TIMP-2 gene in adenoviral particles with PCR. Primers used in PCR reactions were as follows: TIMP-2, sense primer 5'-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3', and antisense primer 5'-GGA AGC TTT TAT GGG TCC TCG ATG TCG AG-3'. PCR was performed in a 50 μL reaction system with 25 cycles (94°C for 20 sec, 65°C for 30 sec, and 72°C for 60 sec). PCR products were subjected to electrophoresis in a 1.0% agarose gel.

3. Results

3.1. *Generation of the shuttle vector pAdTrack-CMV-TIMP-2*

The full length of the TIMP-2 gene was released from the recombinant shuttle vector pAdTrack-CMV-TIMP-2 by *Sal* I/*Xba* I and *Bgl* II/*Xba* I. Sequencing of the TIMP-2 gene yielded 791 bp and was in accordance with what had been previously published in Genbank (Figure 1).

3.2. *Construction of adenoviral vectors pAdTIMP-2 and pAdCMV-GFP for homologous recombination in bacteria*

Zero point five to 1.0 mg of pAdTrack-CMV-TIMP-2 was linearized with *Pme* I and mixed with 0.1 mg of supercoiled pAdEasy-1. Then, electrocompetent *E. coli* BJ5183 cells were added, and electroporation was performed. The cell suspension was then inoculated into 10-cm Petri dishes containing L-agar plus 25 μg/mL of kanamycin. Thirty clones that were kanamycin-resistant were obtained. Smaller colonies (usually representing recombinants) were selected and grown in 2 mL of L-Broth containing 25 μg/mL of kanamycin. Clones were first screened by analyzing their supercoiled sizes on agarose gels and comparing them to pAdEasy-1 controls. The clones that had inserts were further tested by restriction endonuclease digestion, typically with Pac I. As shown in Figure 2, the TIMP-2 gene was detected as a 791-bp diagnostic fragment. Typical digestion of pAdTIMP-2 with *Pac* I yielded the diagnostic fragments

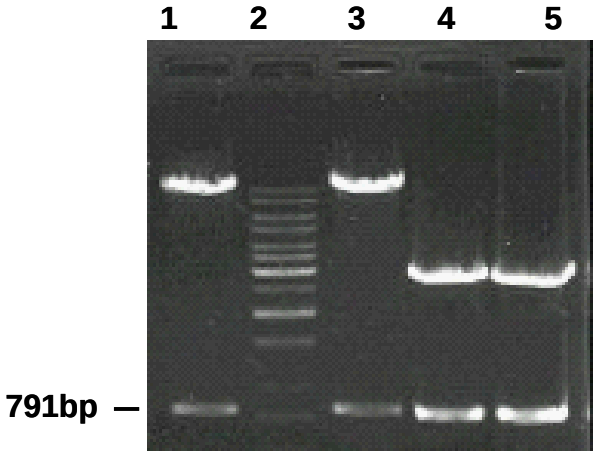

Figure 1. Identification of pAdTrackCMV-TIMP-2. Lane 1, pAdTrackCMV-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 2, DNA ladder; lane 3, pAdTrackCMV-TIMP-2 (linearized with *Bgl* II and *Xba* I); lane 4, pBS-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 5, pGEM-TIMP-2 (linearized with *Eco*RI and *Xba* I).

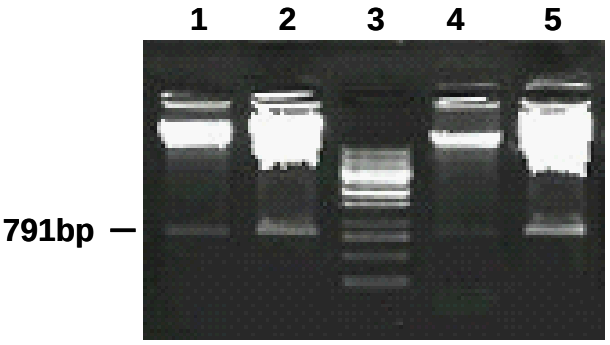

Figure 2. Different clones of recombinant pAdTIMP-2 constructs identified by *Eco*RI and *Xba* I. Some clones in lanes 1, 2, and 5 yielded 791-bp TIMP-2 diagnostic fragments; lane 3, DNA ladder.

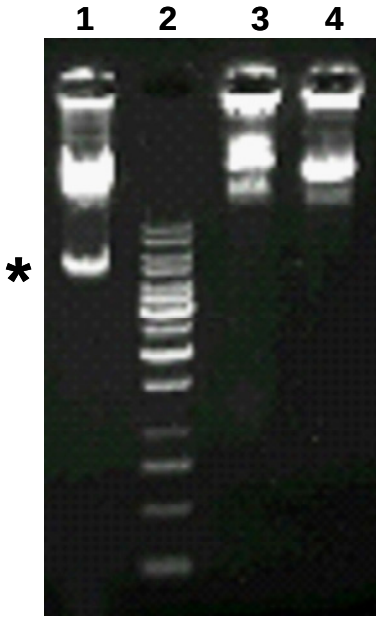

Figure 3. Digestion of pAdTIMP-2 with *Pac* I. Lane 1, pAdTIMP-2 (linearized with *Pac* I); lane 2, DNA ladder; lane 3, pAdMP-2; lane 4, pAdEasy-1.

indicated with an asterisk (Figure 3, lane 1). Figure 4 shows that both the final viral vector pAdTIMP-2 and shuttle vector pAdTrackCMV-TIMP-2 yielded the target gene by restriction endonuclease digestion. The control adenoviral vector pAdCMV-GFP was constructed using the same procedure.

3.3. *Generation of recombinants AdTIMP-2 and AdCMV-GFP*

Transfection of recombinant adenoviral particles in HEK 293 cells was evaluated by tracing the expression of GFP proteins under fluorescence microscopy. As shown in Figure 5, on day 2 after transfection GFP can be seen in about 20% of HEK 293 cells. On day 10, HEK 293 cells were harvested by scraping cells off flasks and pelleted them along with any cells floating in the culture. Finally, the recombinant adenovirus was prepared and purified by CsCl density gradient

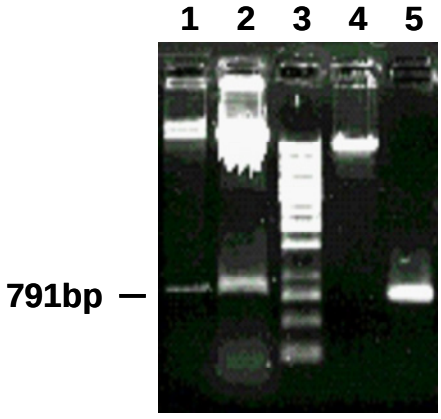

Figure 4. Identification of pAdTIMP-2. Lane 1, pAdTIMP-2 (linearized with *Eco*RI and *Xba* I); lane 2, pAdTrackCMV-TIMP-2 (linearized with *Sal* I and *Xba* I); lane 3, DNA ladder; lane 4, pAdTrack CMV (linearized with *Sal* I and *Xba* I); lane 5, target gene TIMP-2 DNA.

ultracentrifugation as described in Materials and Methods, and its titer was 4×10^{11} pfu/mL. The viral solution was diluted and stored in PBS containing 10% glycerol at −80°C. The control adenoviral vector AdCMV-GFP was constructed *via* a similar method.

3.4. *Identification of TIMP-2 in adenoviral particles by PCR*

After three cycles of freezing and thawing, 5 μL of viral lysate were used for detection of the TIMP-2 gene in adenoviral particles with PCR. At the same time, the shuttle vector AdTrackCMV-TIMP-2 was selected as a positive control. A fragment of 590 bp was obtained, indicating the correct generation of recombinant adenovirus (Figure 6).

4. Discussion

Gene therapy is the transfer of genetic material into somatic cells to effect changes in the pathogenetic processes that contribute to a disease (*9*). Vascular gene therapy has been at the forefront of this field since the first human attempt at gene transfer focused on patients with severe peripheral vascular disease (*10*). Currently, gene therapy techniques are mostly used to treat malignant tumors, and cases of cardiovascular diseases account for about 3 to 17% of all cases of gene therapy, including neointimal hyperplasia or restenosis in vein grafts, arteriosclerosis, peripheral ischaemic vascular disease, and coronary heart disease (*11*).

Gene therapy for vascular disease has particular advantages both in theory and in practice. For example, it can provide long-term expression of a desired protein in tissues and also allows the therapy to target a specific process involved in the disease of interest. In addition, ease of access to the vascular system is another advantage for this type of therapy (*11*).

Replication-defective recombinant adenovirus is an

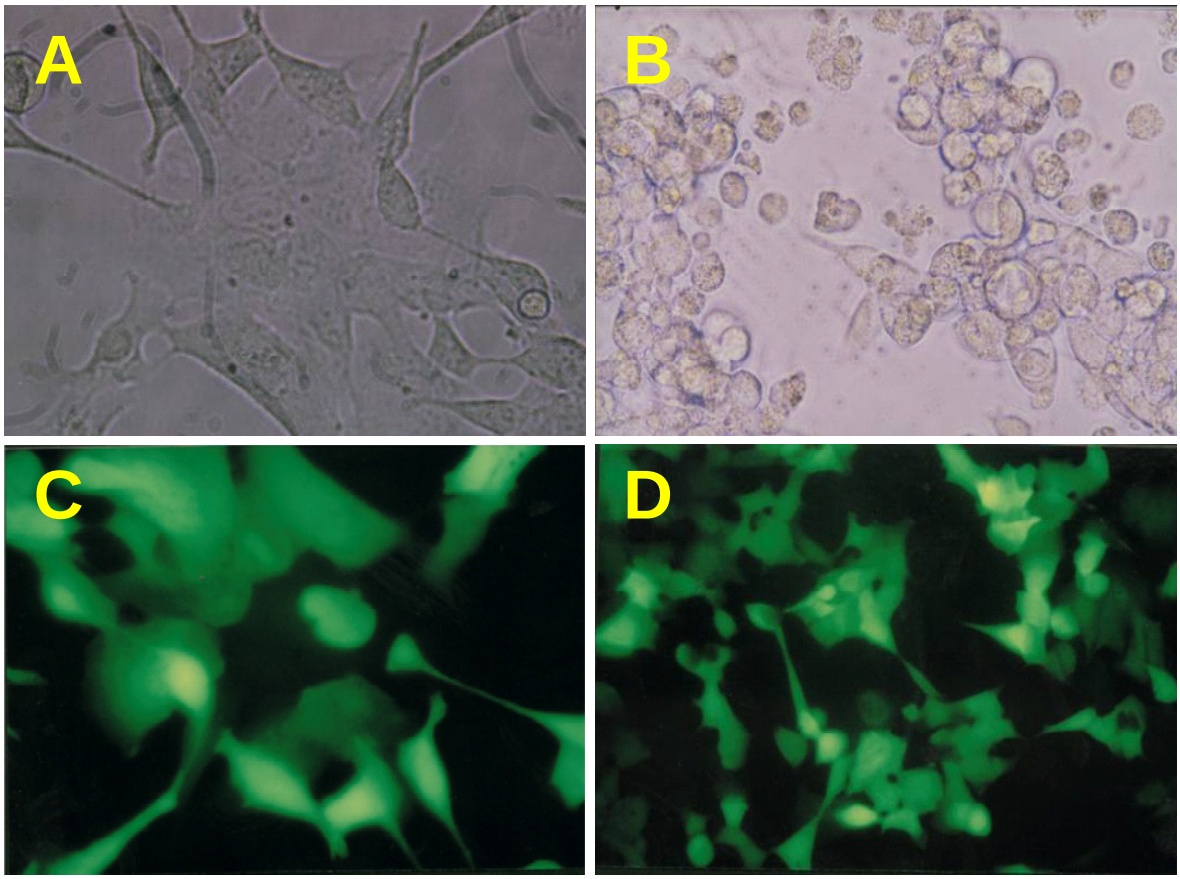

Figure 5. Packaging of recombinant adenovirus AdTIMP-2 in HEK 293 cells. A, normal HEK 293 cells, which were flat and spindly in shape; B, 10 days after transfection of pAdTIMP-2 linearized with *Pac* I into HEK 293 cells, typical comet-like adenovirus-producing foci were observed along with central lysis of cells; C, 2 days after transfection of pAdTIMP-2 linearized with *Pac* I into HEK 293 cells, fluorescence microscopy revealed GFP in about 10~20% of cells; D, 7 days after transfection, GFP was noted in about 50~60% of HEK 293 cells. Over time, the expression of GFP became more marked. A and B, phase contrast microscopy; C and D, fluorescent microscopy. Original magnification, A, B, and D, ×200; C, ×400.

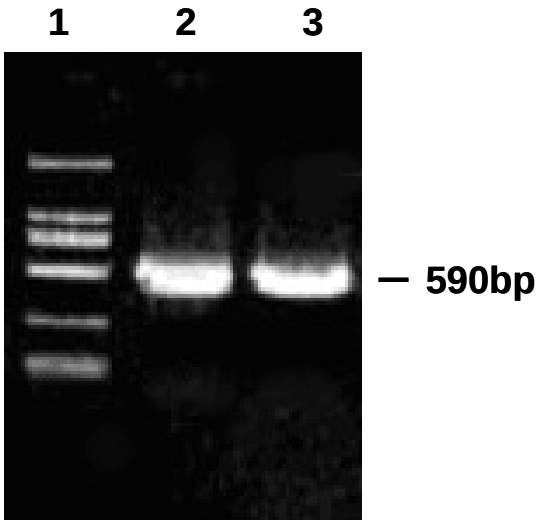

Figure 6. Identification of recombinant adenoviral particles using PCR. Lane 1, DNA ladder; lane 2, AdTIMP-2; lane 4, AdTrackCMV-TIMP-2; A fragment of 590 bp was observed in lanes 2 and 3, indicating the correct generation of recombinant adenovirus.

effective *in vivo* vector system for vascular cell types. It has the advantages of relative ease of penetration into non-dividing cells and allowing preparation of high titer viral stocks (*12*). The conventional method of generating recombinant adenovirus involves homologous recombination in mammalian cells that have the ability to complement defective adenoviruses. Screening individual plaques formed in HEK 293 cells allows the identification of desired recombinants. However, the conventional method has drawbacks in terms of low efficiency of homologous recombination and the time needed for the completion of virus production. Repeated rounds of plaque purification are also needed. In contrast, the AdEasy system of homogenous recombination in bacteria as used in the current study has the advantages of speed and ease. The ability to recover reasonable quantities of homogeneous viruses, without plaque purification, represents another major practical advantage. Furthermore, a GFP tracer is incorporated into the adenoviral backbone, allowing direct observation of all stages of virus production.

Increased proteolysis by MMPs has been reported to be associated with cancer cell invasion, rheumatoid arthritis, and more recently with the neointima formation that characterizes vascular disease (*13,14*). In a previous study of adenovirus-mediated TIMP gene transfer in a cultured human saphenous vein model, TIMP-2 was shown to inhibit neointimal thickening

primarily by inhibiting MMP activity and hence smooth muscle cell migration (*15*). Recently, Xiong *et al*. also found that TIMP-2 promotes aortic enlargement *in vivo*, presumably through its role as a cofactor in the activation of MMP-2 (*16*). The current study successfully constructed a recombinant adenovirus vector carrying the TIMP-2 gene and obtained high titers of virus solution. The study confirmed that the recombinant AdTIMP-2 can be used for further transfection for other cell types.

Replication-defective adenoviruses containing TIMP-2 can now be constructed more easily by homogenous recombination in bacteria than with conventional techniques. The current study has laid the groundwork for further study of gene therapy for vascular disease.

Acknowledgement

This study was supported in part by Japan-China Sasakawa Medical Fellowship of Japan.

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(*Received November 21, 2008; Revised December 19; Accepted December 22, 2008*)

Case Report

Malignant mesothelioma associated with chronic empyema with elevation of serum CYFRA19: A case report

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Summary **Malignant neoplasms are reported to occur with long-standing tuberculous pleuritis or chronic empyema. During the clinical course of chronic empyema, subjective symptoms such as chest pain and deterioration of dyspnea and abnormal clinical signs such as increased abnormal chest shadows have frequently been found. Though difficult, differentiating the occurrence of malignant tumors from worsening chronic inflammation is crucial. We report here a case of malignant mesothelioma associated with chronic empyema with elevation of serum CYFRA19.**

Keywords: Malignant mesothelioma, Chronic empyema, CYFRA19

1. Introduction

The main cause of pleural mesothelioma is exposure to asbestos (*1*). However, malignant pleural tumors can also arise in scars from old tuberculosis, especially after therapeutic pneumothorax, and in chronic empyemas and fistulas (*2,3*). Despite repeated biopsies, detection of malignancy near the empyema cavity is difficult in some cases. Histologically, these tumors are reported to differ somewhat from other mesotheliomas (*4*). Reported here is a case of mesothelioma secondary to asymptomatic chronic empyema with elevation of serum CYFRA19.

2. Case report

An 83-year-old man visited this hospital in February 2006 because a civic health examination indicated an abnormal shadow on a chest X-ray of his left lung. The individual had suffered from tuberculous pleuritis in his 20s. He had also been diagnosed with chronic empyema by another hospital one year prior to this visit. Aspiration cytology from the lower left part of the empyema using a 16 gauge needle revealed no malignancy (data not shown). Chest X-rays revealed an opacity with lobulations in the lower left lung and thickening of the pleura. There were calcified lines (arrowheads) in the basal parts of both lungs (Figure 1a). A chest CT scan revealed a large empyema in the lower left thoracic cavity and calcified pleural thickening bilaterally. Irregular pleural thickening was also found along the left thoracic cage (Figures 2a and b). The patient was diagnosed with old tuberculous pleuritis and chronic empyema. At that time, he had no complaints and was treated as an outpatient. In November 2006, he complained of chest pain and exertional dyspnea, so he was admitted. He had no history of drinking or smoking and had never been exposed to asbestos.

On physical examination, his height was 172.5 cm and his weight was 78 kg. He was not febrile (36.4°C), his pulse rate was 72 /min, and his blood pressure was 123/57 mmHg. There were no heart murmurs. Respiratory sounds were decreased in the entire left lung field. Laboratory tests showed a decreased hemoglobin level (11.3 g/dL), elevated blood sedimentation rate (112 mm/h) and elevated CRP (5.0 mg/dL), though no leucocytosis (5,600 /μL) was seen. Ventilatory function tests showed a %VC of 51.5% and

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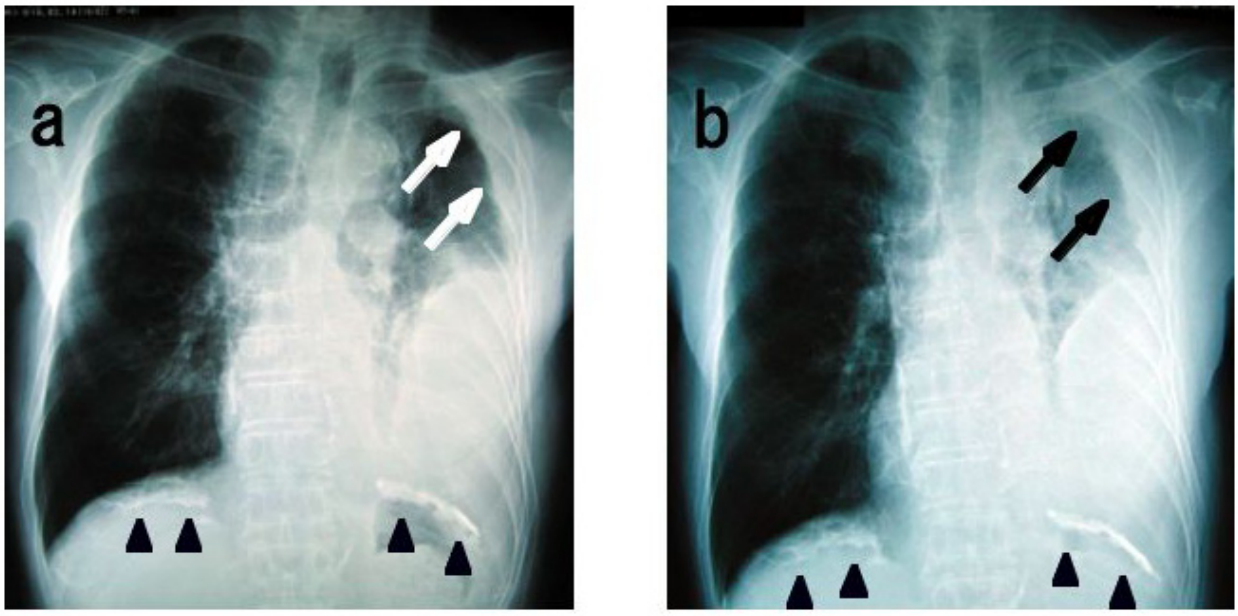

Figure 1. Serial images of chest X-ray. a: A chest X-ray from the first visit shows an opacity with lobulations in the lower left lung and thickened soft tissue density (arrows) along the pleura in the upper lung. There are calcified lines (arrowheads) in the bilateral bases of the lung. *b*: A chest X-ray on admission shows the increased size of the lobulated opacity in the left lung. The upper part of the opacity also has increased thickness along the pleura (arrows) with progressively increasing density in the aerated left lung. Calcified lines (arrowheads) in the bilateral bases of the lung showed no change.

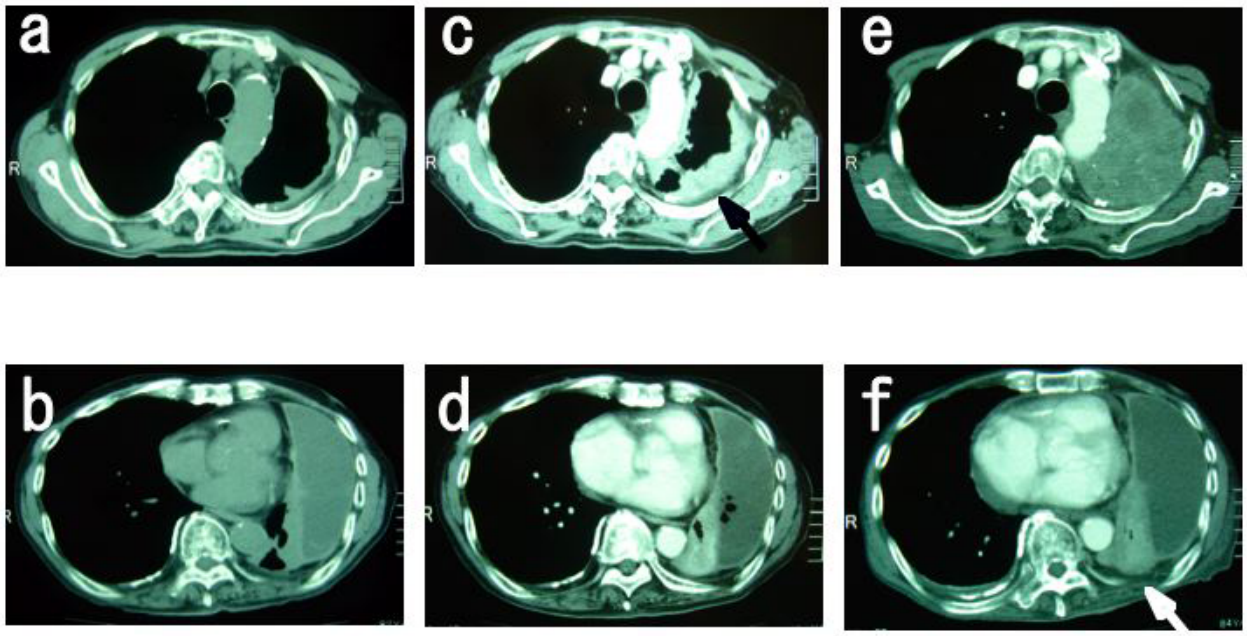

Figure 2. Serial images of chest CT scans. *a* and *b*: Non-contrast chest CT scans on the first visit show pleural thickening along the thoracic cage of the upper left lung with no involvement of the mediastinal side. A large empyema is seen in the lower left thoracic cavity with partial atelectasis of the lower lobe. Some calcifications are observed in the thickened pleura bilaterally. *c* and *d*: Contrast-enhanced CT scans on admission show increased irregular thickening of almost the entire circumference of the pleura involving both parietal and mediastinal sides. The increased size of nodular thickening along the dorsal part (arrow) is 1 cm greater than that in the chest CT scan from the initial visit. The empyema in the left thoracic cavity shows air bubbles probably due to the effect of aspiration cytology. *e* and *f*: Contrast-enhanced CT scans just before death show a fully consolidated upper lung. The size of the empyema is generally consistent but progressive nodular bulging is seen (arrow) in the lower left lung.

a $FEV_{1.0\%}$ of 67.5%. Arterial blood gases (at room air) were as follows: pH 7.45, $PaCO_2$ 41.4 mmHg, PaO_2 90.3 mmHg, O_2 saturation 97.2%. Tumor markers were normal (CEA was less than 0.5 ng/mL, CA15-3 was 5.8 U/mL, CA125 was 32.2 U/mL and NSE was 6 ng/mL). A chest X-ray revealed a diffuse, increased opacity in the entire left lung and thickening of the chest wall over it, indicating deterioration from the chest X-ray during the initial visit (arrows). Calcified lines in the basal parts of both lungs showed no change (arrowheads) (Figure 1b). A contrast-enhanced chest CT scan showed no remarkable change in the chronic empyema in the lower left thoracic cavity but increased irregular thickening of almost the entire circumference of the

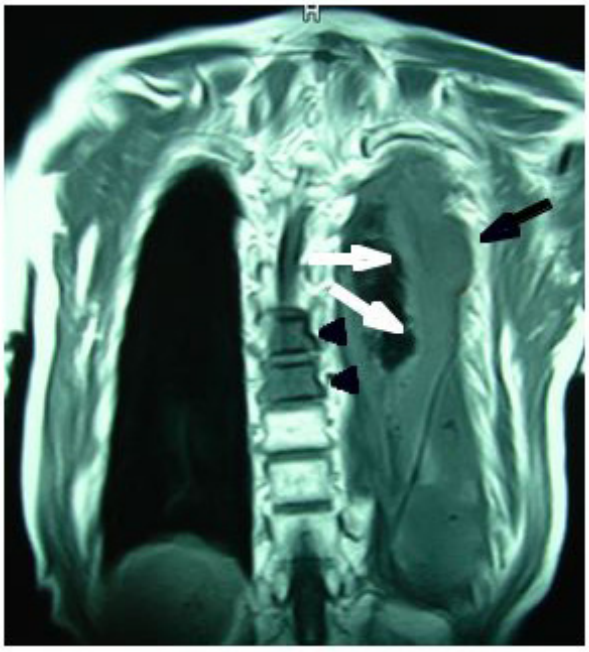

Figure 3. Coronal T1-weighted MR images on admission (SE 500/10) revealed chronic empyema in the lower left thoracic cavity. The irregular mass extends along the pleura and the medial wall of the empyema (arrows). The aerated lung partially is atelectatic with decreased volume. Two thoracic spines (arrowheads) appear as hypointense, suggesting metastasis.

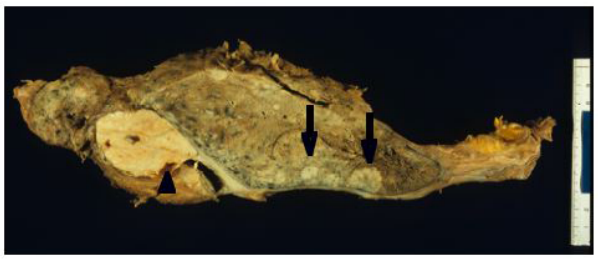

Figure 4. The left lung specimen shows irregular thickening throughout the pleura. Several tumor nodules were seen along with invasion of the lung (arrows). The pleural tumors tightly adhered to the thoracic wall (arrowhead).

pleura (Figures 2c and d). Coronal MR T1-weighted images revealed chronic empyema in the lower left lung and a consolidated mass surrounded by a low-intensity rim (arrows) in the upper left lung (Figure 3).

Aspiration cytology from the soft tissue density lesion along the lower left part of the empyema using a 16 gauge needle again revealed no malignancy (data not shown). CT-guided needle biopsies of the upper thickening pleura were then performed using an 18 gauge core biopsy needle. Pathological examination revealed an epithelial mesothelioma. Immunostaining was positive for anti-calretinin. Immunostaining was also positive for anti-keratin, weakly positive for anti-vimentin, anti-D2-40, and anti-EMA, but negative for anti-CEA (data not shown). Though he was 83 years old, the patient's performance status was good (PS1), and he and his family were eager for treatment, so combination chemotherapy of cisplatin (60 mg/m^2, day 1)/ gemcitabine (80 mg/m^2, day 1, day 8) was given. Chemotherapy was stopped, however, during the second course on account of a high fever. The masses were slightly reduced in size (data not shown). The patient was followed with best supportive care. Chest X-rays while the patient was an outpatient gradually showed deterioration and gradually elevation of the serum level of CYFRA19 (11.5 ng/mL in July 2007 and 59.2 ng/mL in January 2008) while the serum level of CEA remained within the normal range. In February 2008, a contrast-enhanced chest CT scan showed similar findings of chronic empyema in the lower left thoracic cavity as well as a fully consolidated upper left lung (Figures 2e and f). The patient died of mesothelioma in April 2008. Autopsy showed pleural thickening throughout the pleura. Several tumor nodules were seen along the pleura with invasion of the lung (arrows).

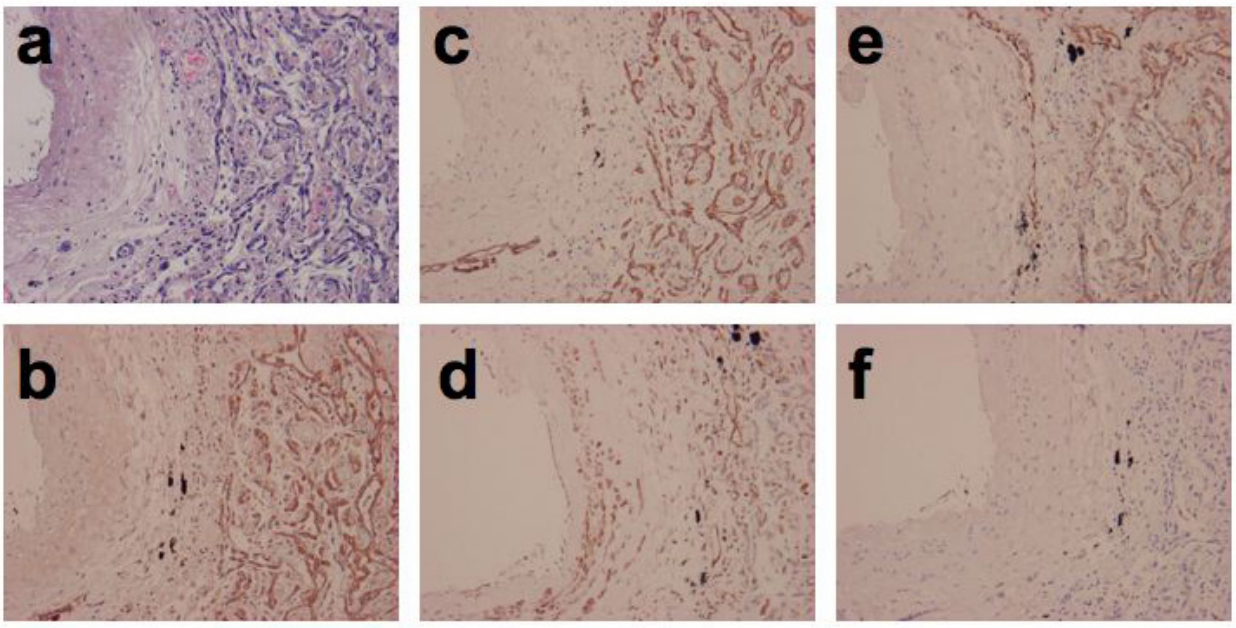

Figure 5. An autopsy specimen of the pleural tumor in the left thoracic cavity shows a pleomorphic tumor (hematoxylin-eosin) that was (*a*) positive for calretinin (*b*), keratin (*c*), vimentin (*d*) and D2-40 (*e*) staining. CEA staining was negative (*f*) (×100).

The pleural tumors tightly adhered to the thoracic wall (Figure 4). Tumor invasion and plaque were seen in the left diaphragm. A massive tumor was noted on the lower surface. Metastatic tumors were seen in the hilar lymph nodes, mediastinal lymph nodes, right lung, stomach, liver, kidneys, spine, and Douglas fossa. Cancerous peritonitis was also seen (data not shown). Autopsy specimens from the pleural cavity of the left lung showed a pleomorphic tumor that was positive for calretinin, keratin, vimentin, and D2-40 and negative for CEA, consistent with malignant mesothelioma (Figure 5). Fibrin and neutrophils were also seen in the autopsy specimen from the pleural cavity of the lower left lung, consistent with chronic empyema, but tuberculosis was not specified as the cause (data not shown).

3. Discussion

Reported here is a case of malignant mesothelioma associated with chronic empyema secondary to tuberculous pleuritis with elevation of serum CYFRA19. The patient had never been exposed to asbestos, and autopsy specimens showed no asbestos bodies, either. Malignant mesothelioma is reported to arise even without exposure to asbestos. Etiologic factors of non-asbestos-related malignant mesothelioma include serious lung diseases, tuberculosis, chemical pneumonia, radiation, and industrial dust and chemicals other than asbestos. A chronic inflammatory process is also suggested as a causative factor, and peritoneal malignant mesothelioma is reported to arise from recurrent peritonitis (*5-7*). The main latency time is reported to be 16 years from the first pneumothorax treatment or signs of empyema to the appearance of symptoms from the tumor (*4*). In this case, the patient was diagnosed with chronic empyema one year before his visit, but his clinical history suggested that he might have suffered from tuberculosis in his 20s. Thus, the latency time in this case may be over 60 years. The mechanism by which mesothelioma occurs due to chronic inflammation is not clear, but involvement of pleural damage and repair is suggested, and persistent inflammation is thought to be required as a factor for malignant mesothelioma (*1*). The Epstein-Barr virus may be implicated in pyothorax-associated lymphoma though not in mesothelioma (*8*).

A differential diagnosis based on malignant pleural disease is extremely difficult in a patient with chronic empyema because malignant mesothelioma has no specific symptoms (*4*). However, deterioration of dyspnea, chest pain, fatigue, and fever are suspected of indicating a malignant disease since these symptoms are uncommon in chronic empyema. In the current case, the patient complained of chest pain and exertional dyspnea on admission. Serum CYFRA19 may have some diagnostic significance. A case of malignant pleural mesothelioma with elevation of CYFRA19 has been reported (*9*). An elevated CYFRA21-1 level with a low CEA level in pleural effusion is also reported to strongly suggest mesothelioma (*10*). In the current case, the serum level of CYFRA19 gradually rose while the serum level of CEA remained negative during the clinical course. Thus, the serum levels of CYFRA19 and CEA may have diagnostic value.

Chest CT scan features suggestive of a malignant pleural disease are reported to be: a pleural rind (specificity 94%, sensitivity 41%), nodular pleural thickening (specificity 94%, sensitivity 51%), parietal pleural thickening greater than 1 cm (specificity 94%, sensitivity 54%), and mediastinal pleural involvement (specificity 88%, sensitivity 56%). On the other hand, a feature suggestive of a benign disease is pleural calcification (specificity 46%, specificity 92%) (*11*). Enhanced chest CT scans reveal "the split pleural sign" and curvilinear enhancement as inflammatory hyperemia of the separated visceral and parietal pleura (*12,13*). On both MR T1-weighted and T2-weighted images, chronic empyema itself is clearly separated by low-intensity rims and shows signal intensities different from those of the tumor (*2*), which coincides with findings in the current case. In a study of radiological evaluation, empyema also displays malignant features, so one or more these findings suggest a high probability of malignant pleural disease (*14*).

Final diagnosis depends on biopsy results, but aspiration biopsies with thin needles are generally useless. Aggressive needle biopsies with large-bore needles or, if possible, incisional biopsies at surgery are recommended (*2*). The diagnosis of malignant mesothelioma is reached by cytology (accuracy 25-30%), blind pleural biopsy (accuracy less than 30%), and CT-guided pleural biopsy (accuracy 85%). Thoracoscopy-guided pleural biopsy (accuracy 98%) can be helpful but invasive, so a CT scan should be performed to reach a diagnosis (*15*). In the current case, the CT findings of a pleural rind and mediastinal pleural involvement on the first visit suggested a malignant disease, but the two aspiration biopsies of the lower chronic empyema revealed no malignancy (data not shown) while needle biopsy of the upper pleural thickening did (data not shown). Malignant tumors associated with chronic empyema are reported to originate in the chest wall around a chronic empyema, and chiefly along the parietal pleura of the empyema (*2*), which coincides with findings in the current case. Thus, this case also suggests the necessity for aggressive needle biopsies and that the biopsy portion of centesis should be carefully decided.

The median survival of patients with malignant mesothelioma from the time of diagnosis is 12 months. Chemotherapy with cisplatin plus gemcitabine results in response rates of 48% and a median survival of 13 months, which represents a significantly longer survival

and better quality of life than with best supportive care (*1,15*). Early chemotherapy is more effective than delayed chemotherapy in symptomatically stable patients. However, there are limited indications for chemotherapy in terms of performance state and age (*16,17*). The current patient survived 16 months from the time of his diagnosis, which was 3 months longer than the median survival in months. Chemotherapy with cisplatin plus gemcitabine might have contributed to his survival, though it was stopped after the first course.

In conclusion, chronic empyema can induce malignant mesothelioma over a long clinical course. CT scans are recommended even in asymptomatic patients with chronic empyema at least every year. The elevated serum level of CYFRA19 and the normal serum level of CEA may also have diagnostic value. Particularly in the case of CT findings suggestive of malignant disease, physicians should not hesitate to perform more invasive examinations such as a core needle biopsy to make a diagnosis.

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(*Received December 13, 2008; Accepted December 15, 2008*)

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