including nursing science, nursing care, study of social welfare, social science, psychology, economics, religion and ethics, as well as medical sciences;
2 Promotion of gerontology, reform and enhancement of geriatrics in undergraduate, postgraduate and lifelong education;
3 Building geriatric medical centers in each area, and accumulating large-scale evidence of geriatric diseases and geriatrics; and
4 Structural development and promotion of home-based care and multidisciplinary care.
Through implementation of the above measures, Japan is expected to function as a successful example for the rest of the world. **Geriatr Gerontol Int 2012; 12: 16–22.**

Keywords: education, elderly, geriatrics, gerontology, multidisciplinary approach.

1. Preface

Over the past 50 years, the percentage of elderly people in the population of Japan has increased fourfold from 5.7% in 1960 to 23.1% in 2010. Japanese society is aging at an unprecedented rate. According to the National Institute of Population and Social Security Research, the percentage the elderly population is estimated to continue increasing, reaching 26.0% in 2015 and further increasing rapidly. After 2020, the percentage of elderly people in the population is expected to stabilize; however, as a result of a decrease in the total population, the percentage will further increase to 40.5%, peaking in 2055. Japan will face a super-aged society, in which 40% of the population will be over 65 years-of-age. Unless appropriate countermeasures are taken, such as a rapid improvement in clinical skills and knowledge among physicians involved in geriatrics, marked advances in the prevention of lifestyle-related diseases, prevention of geriatric syndromes including dementia, and marked expansion of home-based care or local-care, we cannot avoid a situation where many frail elderly people have to live with no support. However, many issues remain; that is, a marked reduction of long-term care facilities, a reduction in length of hospital stay in acute hospitals and a delay in expanding home-based care system, and whether thanatology reflects a social change. We should also consider social issues, such as ageism, caregiver burnout, dignified death and the appropriateness of placing gastrostomy tubes in elderly patients with dementia. To provide dignified care, particularly for older people, appropriate care should be carried out in not only the terminal phase, but also during the last few years before death.

However, despite the challenge, little is known about gerontology and geriatrics in Japan, and they are not fully used in clinical settings or education. To solve this problem, a macroscopic integration and cooperation are needed, using an interdisciplinary approach involving medical science, nursing science, nursing care, study of social welfare, social science, engineering, jurisprudence, economics, psychology and ethics. Furthermore, along with the reform and enhancement of geriatrics in undergraduate and postgraduate education, fostering specialists who can practice geriatrics is needed. Also, for non-geriatricians or general practitioners who currently and prospectively provide care in clinical settings, an educational system should be prepared to deepen their understanding of geriatric medicine.

2. Current situation and measures

(1) Social contribution of the elderly and the medical economy

As a result of the low birth rate, the percentage of the total labor force (aged 20–64 years) is expected to decrease in Japan. Elderly people are usually divided into two groups based on age: 65–84 years (young-old) and 75 years and older (old-old). Although many elderly people, particularly the young-old, have sufficient physical strength to fulfil their job duties and a make social contribution though productive activity, they are not fully utilized. The promotion of social participation and the contribution of the elderly is expected to contribute to creating purpose in their lives, as well as an increase of a substantive productive population, financial stability and self-sustainability for the elderly, and an upturn of national economic activity through an increase of total consumption. Therefore, for elderly people to be engaged in various social activities, strategies for developing a social structure for re-education, volunteer activity, various employment statuses and employment opportunities should be prepared using an interdisciplinary approach involving study of social welfare, social science and economics. However, as the total number of jobs is fixed, consideration should also be given to young workers.

Life expectancy in Japan is the highest in the world. Japan also has the highest healthy life expectancy. In 2008, USA health expenditures accounted for 16% of the nation's gross domestic product (GDP), twice the Japanese rate. Compared with other countries, Japanese health expenditures as a percentage of GDP accounted for two-thirds of that of France and Germany, suggesting that we have the most cost-effective health-care

systems. In addition, the annual cost of health care has been approximately 670 000 yen per elderly person for the past 10 years. However, the aging of the population is expected to impact on future spending growth. Sasaki compared life-long medical costs between the longevity and non-longevity groups, and found that longevity decreases medical costs and has positive economic impacts.[1] Thus, it is important to enhance preventive medicine to achieve longevity, make continuous efforts for cost-effective medicine and improve satisfaction with the health-care systems. Discussion of geriatric medicine should be made after disclosing the aforementioned facts to the public.

Problems in geriatric medicine are closely linked to social structures, including care, welfare and dwelling surrounding the health-care system. To reveal and solve problems regarding the elderly and an aged society, the promotion of gerontology using an interdisciplinary approach is increasingly needed.

Regarding employment opportunities for older workers and future directions of medicine, care and welfare, discussion should be made among specialists from various health-care specialties. The Japan Geriatrics Society and the Japan Gerontological Society, as a core organization, should expand their activities to achieve a "society where elderly people can enjoy their lives" with the cooperation of the National Center for Geriatrics and Gerontology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, the Institute of Gerontology the University of Tokyo, and J. F. Oberlin University.

(2) The current state of geriatric medicine and its direction

Geriatric disorders have several features.

First, diseases occur as a result of a decline in organ systems associated with aging. Therefore, even if a disease is not so severe, a patient might have been developing an unexpectedly marked decline in organ systems. In addition, homeostatic function with aging, biophylaxis capacity and nutritional absorption capacity often decrease, and symptoms become chronic and refractory.

In terms of clinical symptomatology, older people often complicate many diseases together with a geriatric syndrome with multiple etiologies. Signs and symptoms vary according to each individual, and are often atypical. Response to drugs is different in elderly compared with non-elderly people.

Older people are more likely to develop multiple diseases, and visit different hospitals and receive many screening tests and prescriptions at the same time;[2] thus, total expenditures on the elderly become inevitably high, which has been said to cause financial collapse of the Japanese health insurance system. However, regarding this issue, we should focus on the medical cost required for a single disease between elderly and non-elderly people, and we should be aware that restricting the increasing financial burden on patients to receive screenings or prescriptions for each disease would be ageism for elderly people and uncontroversial. However, unnecessary duplication of the screening given at each hospital should be avoided. To achieve this, an effective screening system carried out by primary-care physicians, and privacy-preserving medical data sharing of test results and medication among hospitals and clinics are needed. Regarding medications, the Japan Geriatrics Society has prepared the "Guidelines for medical treatment and its safety in the elderly" as an outcome of the sponsored research in Japan Foundation on Aging and Health.[3] The guideline explained standard medical treatments mainly for the elderly by giving examples of low priority, such as making an easy prescription or non-evidence-based prescription to prevent deterioration of chronic disease. In either retrospective fee-for-service or a prospective payment system (fixed amount), physicians should provide the same level of prescription to each patient. To carry out effective screening for the elderly or evidence-based medical treatment, a constructive research system should be developed separately from health-care reform in terms of medical economy. The Japanese government has decided to abolish the existing medical insurance system for those aged 75 years and older; however, the following principles stated in the existing medical insurance system should be included in the next system for the elderly: (i) elderly disease prevention; (ii) comprehensive geriatric assessment; and (iii) incentives to promote discharge planning.

Older people often develop functional disorders associated with chronic disease or aging. Functional disorders not only jeopardize the independence of people and pose social disadvantage, but also lead to secondary disease. This often makes elderly people fully dependent, resulting in lower quality of life. Therefore, in the treatment of geriatric disorders, priority should be given to functional outcomes, as well as life expectancy and the prognosis of organ systems. In addition, because a psychological change associated with an environmental change often leads to a deterioration of symptoms in elderly people, treatment policy and discharge planning should be prepared with a holistic consideration of the patient using the comprehensive geriatric assessment (CGA). In geriatric medicine, it is important not only to protect organ systems, but also to maintain physical function to prevent assisted living.

To maintain independent living, a person needs to have sustained function, including daily life functions, cognitive function, emotion and sociality (family, friends, job). CGA is used to determine the aforementioned functional status both comprehensively and systematically. The results of CGA give us a clue of what kind of

support can help maintain independent living or assisted living with minimum care for elderly people. However, CGA is not a popular tool. Therefore, we should examine ways of increasing the awareness of CGA to promote its use for the improvement of geriatric medicine.

End-of-life care for elderly patients is an extremely important issue in geriatric medicine; however, very few elderly people in Japan have made advance directives to show their wishes about their health care during the end-of-life period. In geriatrics, there are so many issues to discuss, including confirmation of patient's wishes, the need of a health-care representative, and the relationship between the patient and their physician. Therefore, we should investigate the awareness of end-of-life care for elderly patients among health-care professionals, including physicians and nurses, people involved in care, patients, and their families, to discuss future direction of care. Regarding end-of-life care in elderly people, "Attitudes toward end-of-life care in elderly patients",[4] which was announced in 2000 by the ethics committee of the Japan Geriatrics Society and is currently under revision, and a proposal prepared by the end-of-life care research group,[5] should be referred.

(3) Fostering health-care professionals involved in geriatric medicine

Despite the growth of the elderly population, physicians with special geriatric training are not expected to increase under the present system of medical education. In order to solve the problem of care for the growing elderly population, the educational system should be restructured to provide an understanding of geriatric medicine for non-geriatricians, general practitioners and physicians working at care facilities that provide care for elderly patients. This might be an effective and practical approach for fostering physicians taking care of the elderly. To provide sufficient geriatric knowledge to general practitioners and non-geriatricians, the education program should include basic geriatrics contents to retain quality of geriatric care, which would be required even for non-geriatricians. The Japan Geriatrics Society has published *Clinical Handbook for Active Aging and Geriatric Care* for physicians, which aims to provide basic knowledge of elderly-specific symptoms, assessment, treatment and care. It is expected that using this handbook for students, residents, practitioners and non-geriatricians might contribute to the expansion of geriatric medicine. In the USA, in order to deal with a shortage of geriatric specialists, medical students are required to receive a minimum geriatrics education.[6]

(4) Promotion of geriatric disease clinical research

In Japan, a system for making diagnosis and providing treatment and care for patients with elderly diseases, including dementia, has not been fully developed. In elderly care, it is important to make an accurate diagnosis and collect clinical evidence to reflect diagnosis and evidence in clinical settings. To accumulate evidence of geriatric medicine and nursing, the promotion of clinical research and a marked expansion of geriatric medical centers with high-level medical services are eagerly awaited.

Currently, there are just two geriatric medical centers in Tokyo and Nagoya. Therefore, the number of centers should be increased and should be placed in each district (Hokkaido, Tohoku, Hokuriku, Kanto, Koshinetsu, Tokai, Kinki, Chugoku, Shikoku and Kyushu). The National Center for Geriatrics and Gerontology, as a core facility, is required to examine the efficacy of geriatrics-related activities and consistency with countermeasures, supervise multicenter studies and clinical research projects, and strive to enhance geriatric medicine through the standardization of geriatric medicine and care, and preparation of medical guidelines. In this process, each center, as a platform of geriatric medicine, should accumulate clinical data, and is also required to function as a facility to educate non-geriatricians.

The Japan Geriatrics Society has been carrying out clinical research on the treatment of hyperlipemia involving the elderly aged 75 years and over. An establishment of a support system for such clinical research and an accumulation of evidence on the efficacy of nutrition and exercise are also considered important.

(5) Promotion of home-based care and multidisciplinary care

Based on the demand of older people who prefer to remain at home, and a government policy that aims to shorten the length of hospital stay and the number of beds to decrease the growing burden of health-care expenditure, the promotion of home-based care has been provided. However, the medical structure of home-based care has not been fully devised, requiring further development of a medical and nursing structure where older people can receive continuing treatment and care, including rehabilitation, within the local community, while not being too dependent on the hospital stay, or not being forced to choose home-based care. Enhancement of home-based care might contribute to reducing the burden on physicians and nurses at acute hospitals, and might also compensate for other care services, such as emergency care and obstetrics.

One of the concerns of home-based care among physicians, patients and their families is the difficulty with hospital admissions in the event of sudden illness or deterioration. To solve this problem, the National Center for Geriatrics and Gerontology has established a "Home-based care unit". Preregistration from both a general practitioner and the patient is necessary for

admission to this unit, with the intention to continue home-based care. The patient can be admitted any time by referral of a general practitioner. The outcome of this program is eagerly awaited.

In home-based care settings, a group of professionals from diverse disciplines mutually cooperate to provide care for a patient. For such a multidisciplinary approach, it is important to choose appropriate professionals according to the condition and disease stage of the elderly patient. However, this multidisciplinary approach involves some problems. One is the legislative "gap" between health-care providers registered under the Medical and Dental Practitioners Acts and the Act on Public Health Nurses, Midwives and Nurses, and nursing care providers registered under the Long-Term Care Insurance. The other is the discrepancy in the principle between health-care and nursing-care providers. To solve these problems, it is essential to examine them along with the legislative issues, and promote home-based care, particularly at universities offering courses in geriatrics and local community hospitals where there are accumulating results of a multidisciplinary approach to caring for elderly patients, to further promote the cooperation between medical-care and social-welfare services.

3. Proposals

We make the following proposals as countermeasures against various issues in geriatrics:

(1) Development and promotion of a system that enables elderly people to participate socially and make a contribution using an interdisciplinary approach among the various areas, including nursing science, nursing care, study of social welfare, social science, engineering, psychology, economics, religion and ethics, as well as medical sciences.

Promotion of social participation and contribution of the elderly, while considering the total number of jobs and young workers, is expected to contribute to creating purpose in their lives, and reduce the growing number of older people who become frail or in need of care. It is also expected to bring about an increase in a substantial productive population, financial stability and self-sustainability for the elderly, and an upturn of the national economic activity through an increase of total consumption.

(2) Promotion of gerontology, reform, and enhancement of gerontology and geriatrics in undergraduate, postgraduate and lifelong education.

To solve problems associated with elderly people or an aged society, gerontological and geriatric research and education should be enhanced. By fostering medical professionals who understand the physical and mental traits of older adults, and those who can provide a holistic approach with consideration to organic integration with nursing care, provision of reliable care and nursing services is expected.

(3) Build geriatric medical centers in each area, and accumulate large-scale evidence of geriatric diseases and geriatrics.

For system reform of diagnosis, treatment and nursing care, evidence should be accumulated through large-scale clinical studies.

(4) Structural development and promotion of home-based care and multidisciplinary medicine and care.

Promotion of home-based care and multidisciplinary medicine and care, particularly at universities offering courses in gerontology and local community hospitals where there are accumulating results of a multidisciplinary approach to care for elderly patients, can be expected to help reduce the burden of physicians and nurses, and meet the demand of older people.

Through implementation of the aforementioned measures, Japan is expected to function as a successful model for the rest of the world.

4. Summary

The phenomenon of an aging population is often considered within a negative spectrum; however, elderly people in need of care only account for 13% of the total elderly population, and this is not being expected to further increase. We should rather focus on the fact of an increasing number of "healthy elderly individuals with rich experience and knowledge", which would not become a negative factor in the future. The restructuring of these healthy elderly resources for social development is believed to bring a permanent bright future, and it is expected that medical-care and social-welfare services will make a significant contribution within this framework. The realization of healthy longevity in society is possible; however, we should be aware that it is only possible by the integration of geriatric medicine and social welfare.

To cope with the problems that come with a rapidly aging society as the world-leading model, the development of elderly-friendly medical devices and nursing-care equipment to avoid a labor shortage is considered essential. Taking the lead in the development of medical equipment for elderly people enables us to provide other countries with aging populations with a model for success, and is also expected to contribute to the creation of new employment and an increase in export as one of the main industrial products in Japan.

The task given to the country with the longest healthy life expectancy is to try to achieve the highest level of elderly satisfaction. As a result of a community change, "roles" and "presence with respect" of the elderly have become weakened, and a medical- and nursing-care "burden" for the younger population has been casting

a dark shadow over the society. As the baby boomer generation ages into elderly status, new roles, including a future health-care workforce and volunteer activities, and community satisfaction should be rebuilt. Gerontology and geriatrics ought to take the lead in showing a practical approach to the industry and the administration to create new images of the elderly.

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Disclosure statement

The authors declare no conflict of interest.

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Src kinase-mediates androgen receptor-dependent non-genomic activation of signaling cascade leading to endothelial nitric oxide synthase

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ABSTRACT

Our previous study has demonstrated that testosterone rapidly activates endothelial nitric oxide synthase (eNOS), enhancing nitric oxide (NO) release from endothelial cells (ECs) via the phosphatidylinositol 3-kinase/Akt (PI3-kinase/Akt) pathway. The upstream regulators of this pathway are unknown. In this study, we further investigated the non-genomic action of testosterone in human aortic ECs. Acute (30 min) activation of eNOS caused by testosterone was unaffected by pretreatment with a transcriptional inhibitor, actinomycin D. Non-permeable testosterone-BSA rapidly induced Akt and eNOS phosphorylation. In contrast, luciferase reporter assay showed that the transcriptional activity of the androgen-responsive element (ARE) was increased by testosterone, but not by testosterone-BSA at 2 h after stimulation. Immunostaining displayed co-localization of androgen receptor (AR) with caveolin-1. Fractional analysis showed that AR was expressed in caveolae-enriched membrane fractions. Immunoprecipitation assays revealed the association of AR with caveolin-1 and c-Src, suggesting complex formation among them. Testosterone rapidly increased the phosphorylation of c-Src on Tyr416, which was inhibited by an AR antagonist and by siRNA for AR. PP2, a specific-inhibitor of Src kinase, abolished the testosterone-induced phosphorylation of Akt and eNOS. Our data indicate that testosterone induces rapid assembly of a membrane signaling complex among AR, caveolin-1 and c-Src, which then facilitates activation of the c-Src/ PI3-kinase/Akt cascade, resulting in activation of eNOS.

1. Introduction

Steroid hormones play various roles in vascular functions through specific receptors localized in vascular endothelial cells (ECs) or vascular smooth muscle cells [1,2]. The production of the vasodilator nitric oxide (NO) by endothelial NO synthase (eNOS) is a key mediator of endothelial homeostasis, including normal vasomotor function [3]. Several lines of evidence indicate that testosterone can exert acute endothelium-dependent vasodilator effects upon various vascular beds and tissue perfusion throughout the body [4,5], and that these effects of testosterone are mediated in part by NO [6]. However, the detailed mechanisms of rapid vasodilatation by testosterone are unknown.

The androgen receptor (AR) is a member of the steroid nuclear receptor super-family, which exerts its effects by modifying gene expression [7–9]. In addition to its canonical genomic action, AR also exhibits acute actions, designated as non-genomic actions, which take place in a membrane-delimited signal pathway, taking only several seconds to minutes [10–12]. In vascular ECs, however, the range of signal transduction pathways activated by membrane AR has not been defined, and the potential roles of these pathways to mediate testosterone actions in vascular cells are largely unknown.

In vascular ECs, it is now established that eNOS and other regulatory proteins are co-localized in specialized signal-transduction plasma membrane domains, caveolae [13,14]. The localization of eNOS in caveolae is required for its activity [15,16]. We previously have demonstrated that in vascular ECs, testosterone acutely stimulates rapid eNOS activation and enhances NO production via activation of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt

Abbreviations: AR, androgen receptor; ARE, androgen-responsive element; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; PBS, phosphate buffered saline; PI3-kinase, phosphatidylinositol 3-kinase; testosterone-BSA, bovine serum albumin-coupled testosterone; NO, nitric oxide; siRNA, small interference RNA; NT-siRNA, non-targeting scrambled siRNA; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*] pyrimidine.

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cascade driven by a direct interaction between AR and the p85α subunit of PI3-kinase [17]. In the present study, we further investigated whether testosterone activates eNOS via a membrane-AR mechanism, i.e., a non-genomic action, in human aortic ECs. We also investigated the upstream regulators involved in eNOS activation in the caveolae molecular complex.

2. Materials and methods

2.1. Cell culture

Human aortic ECs were maintained in EBM-2 (Clonetics) medium supplemented with 10% FBS and a growth factor cocktail as previously described [17]. Cells were used for the present experiments in the 5th and 7th passages. In the experiments using bovine serum albumin-coupled testosterone (testosterone-BSA) (Sigma), testosterone-BSA was diluted in culture medium to the indicated concentrations, then mixed with dextran-coated charcoal (50 mg/ml) for 30 min at room temperature, centrifuged at 3000*g* for 15 min and passed through a 0.2-μm pore size filter to remove any potential contamination with free testosterone. In inhibitor experiments, inhibitors were added 60 min before cells were treated with testosterone (100 nM).

2.2. Transfection of plasmids and luciferase reporter assay

The pGL3 vector containing two copies of androgen-responsive element (ARE) upstream of the minimal thymidine kinase promoter ligated to a luciferase reporter gene (2× ARE-TK-pGL3-luc) was used for luciferase reporter assay as previously reported [18]. As an internal control of transfection efficiency, a renilla luciferase plasmid pRL-TK (Promega) was co-transfected. Cells were seeded in six-well plates in culture medium and grown until 50–60% confluence, then transfected with the 2× ARE-TK-pGL3-luc reporter plasmid and pRL-TK control prasmid using SuperFect transfection reagent (Qiagen) for 24 h according to the manufacturer's instructions. Then, cells were rinsed with Hank's balanced salt solution buffer (Sigma) once, starved and exposed to testosterone (100 nM) or testosterone-BSA (100 nM) for an additional 2 h. The amount of plamid DNA of 2× ARE-TK-pGL3-luc per well was adjusted up to 0.9 μg and that of pRL-tk was 0.3 μg, as an optimal microgram ratio of 2× ARE-TK-pGL3-luc to pRL-TK was determined to be 3:1. The optimal ratio of the total amount of plasmid DNA (μg) to SuperFect transfection reagent (μl) was determined to be 10:1 by supplemental experiments. Firefly and renilla luciferase activities were measured using a dual luciferase assay system (Promega) according to the manufacturer's instructions. The firefly luciferase values of each sample were normalized by renilla luciferase activity, and data were reported as relative light units.

2.3. Immunostaining

Cells were plated onto type I collagen-coated cover slides and grown in culture medium until confluence. Cells were then fixed in 2.5% paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature. After washing with cold PBS 3 times, cells were blocked with 3% milk for 30 min to prevent nonspecific binding. Slides were then incubated with a primary antibody mixture of rabbit anti-AR (N-20) (1:50 dilution; Santa Cruz) and mouse anti-caveolin-1 (1:100 dilution; BD Transduction) overnight, and then washed three times with PBS followed by incubation with a secondary antibody mixture of Alexa-fluor 488 goat anti-rabbit secondary Ab (1:200 dilution; Invitrogen) and Alexa-fluor 555 anti-mouse secondary antibody (1:200 dilution; Invitrogen) for 2 h. Cells were then incubated for 5 min with DAPI (Dojindo) to stain nuclei. Slides were mounted using mounting medium (Dako), and visualized using an Olympus FV300 laser scanning confocal microscope.

2.4. Cell fractionation

Low-density, caveolae-enriched membrane fractions were isolated using a Caveolae/Rafts Isolation kit (Sigma) according to the manufacturer's instructions. Cells were grown in 60-mm dishes until confluence, harvested in ice-cold lysis buffer containing 1% Triton X-100 and protease inhibitor cocktail (1:100 dilution), and sonicated for 5 s. The resulting cell lysates were cleared by centrifugation for 5 min. Supernatants were mixed with OptiPrep gradient layers, and the mixtures were obtained by centrifugation at 200,000*g* for 4 h using a Beckman SW70.1Ti rotor, and then analyzed by collecting and numbering 9 fractions (each 1 ml) from the top of the tubes. These fractions were subjected to immunoblotting. The amounts of all fractions of the loaded samples were equalized before gel electrophoresis. OptiPrep density gradient and immunoblotting experiments were performed four times, and representative blots are shown.

2.5. Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting experiments were performed as previously described according to standard protocols [17]. Antibodies against AR (N-20) for immunoblotting, AR (441) (Santa Cruz) for immunoprecipitation, phospho-eNOS^{Ser1177} and eNOS/NOS type III (BD Transduction), phospho-Src^{Tyr416} (Cell Signaling), c-Src (B-12) (BD Transduction), caveolin-1 (Sigma) for immunoblotting and caveolin-1 (BD Transduction) for immunoprecipitation were used. In all immunoprecipitation and immunoblotting experiments, blots were performed three times, and representative blots are shown. In some experiments, densitometry analysis was performed using an image scanner and analyzing software (NIH image Ver. 1.61).

2.6. Small interference RNA (siRNA) transfection

siRNA duplex against AR (Santa Cruz; Accession No: sc-29204) was used for directed knock down of AR expression. Non-targeting scrambled siRNA (NT-siRNA) (Santa Cruz; Accession No: sc-37007) was used as control siRNA. Transfection of siRNA was performed as previously described [17].

2.7. Statistical analysis

Values are expressed as mean ± SEM in the text and figures. Statistical comparisons were performed using ANOVA with post hoc Fisher's protected least significant difference test. Differences with a value of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Testosterone induced eNOS activation via non-transcriptional mechanisms

In our previous study, we demonstrated that ECs contain the AR, which is involved in phosphorylation of eNOS induced by testosterone [17]. To examine whether the response to testosterone is triggered through the nuclear receptor or initiated at the plasma membrane receptor in ECs, firstly, we investigated whether testosterone-induced activation of eNOS would be affected by exposure to actinomycin D (10 μM), a transcriptional inhibitor. As shown in Fig. 1A, acute (30 min) activation of eNOS caused by testosterone

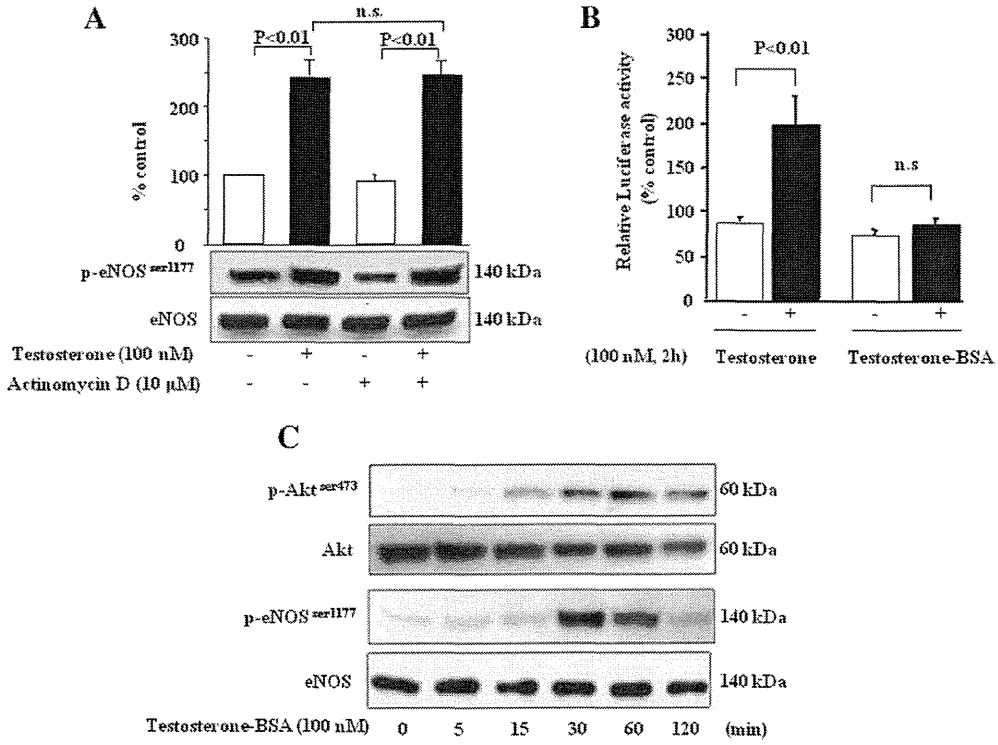

Fig. 1. Testosterone induced eNOS activation via non-transcriptional mechanisms. (A) Steroid-deprived, serum-starved ECs were pretreated with or without actinomycin D (10 μM) for 60 min before exposure to testosterone. Then, cells were treated with vehicle (0.01% DMSO) or testosterone (100 nM) for 30 min. Phosphorylation of eNOS at Ser1177 (p-eNOS^{Ser1177}) and the total eNOS level in cell lysates were analyzed by immunoblotting and densitometric analysis. Data represent mean ± SEM of the p-eNOS/eNOS ratio of quantified densities from three independent experiments. Representative blots are shown. (B) Luciferase reporter assay of ARE. Cells were transfected with the indicated plasmids in culture medium for 24 h, then starved and exposed to testosterone or testosterone-BSA for 2 h. After cells had been washed and harvested, cell lysates were prepared and used for luciferase reporter assay as described in Section 2. The results were obtained from three sets of transfection and are presented as mean ± SEM. $n = 6$. (C) Cells were treated with testosterone-BSA or vehicle for the indicated times. Phosphorylation of Akt and eNOS or the total levels of Akt and eNOS in cell lysates were analyzed by immunoblotting. The experiments were performed at least three times with comparable results.

was unaffected by pretreatment with actinomycin D. Then, we used membrane-impermeable testosterone-BSA to investigate whether testosterone triggered eNOS activation is mediated by the cell membrane AR. In the luciferase reporter assay, transcriptional activation of a specific DNA-binding response element, ARE, was increased when the cells were exposed for 2 h to testosterone, but not by exposure to testosterone-BSA (Fig. 1B). In contrast, testosterone-BSA rapidly induced Akt and eNOS phosphorylation (Fig. 1C), as is the case of testosterone [17]. These data indicate that testosterone rapidly activates eNOS via non-transcriptional mechanisms.

3.2. AR was distributed to caveolae in response to testosterone

As a signal complex scaffold protein, caveolae have been postulated to organize and modulate signal output [14,19]. Although AR is predominantly expressed in nuclei, confocal double immunostaining showed the co-localization of caveolin-1, a caveolae marker, and AR (Fig. 2A). In parallel, we performed cell fractionation with equilibrium density gradient centrifugation to determine whether AR is expressed in the caveolae-enriched membrane fractions. Caveolin-1 was highly concentrated in lighter fractions (Fig. 2B, #3 to #6 fractions), indicating that fractions 3–6 are the major caveolae-like plasma membrane microdomains under our experimental conditions. Although the majority of AR was deposited in the higher-density fractions (#7 to #9 fractions), a significant amount of AR was detectable in caveolin-1-enriched membrane fractions.

Next, we examined whether AR interacted with caveolin-1 directly in response to testosterone. As shown in Fig. 2C, the association of AR with caveolin-1 was detected in immunoprecipitation complex by immunoblotting reciprocally and significantly increased by exposure of cells to testosterone for 30 min, while the complex was detected at a low level without testosterone treatment. These results indicate that cells membrane AR was redistributed to the caveolin-1-enriched membrane fractions in response to testosterone.

3.3. c-Src kinase is a critical upstream regulator in PI3-kinase/Akt activation cascade

The Src family consists of nonreceptor tyrosine kinases that include nine members such as Src, Yes, Fyn, and c-Fgr. In some cells, Src is a critical upstream regulator of steroid-stimulated membrane signal transduction pathways [13,14]. In cell fractionation experiments, c-Src was present in the caveolin-1-enriched membrane fractions isolated from ECs (data not shown). We next investigated whether Src kinase mediates testosterone-induced eNOS activation in ECs. Phosphorylation of c-Src on Tyr416, the active form of c-Src, was increased after 5-min treatment of cells with testosterone (Fig. 3A). Pretreatment of cells with 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d] pyrimidine (PP2; Sigma), a Src family kinase specific inhibitor, blocked the testosterone-induced phosphorylation of Src (Fig. 3A). PP2 also abrogated testosterone-stimulated Akt and eNOS phosphorylation (Fig. 3B). These data indicate that c-Src kinase is a critical upstream regulator of the Akt phosphorylation cascade for eNOS activation in ECs.

Testosterone-induced phosphorylation of c-Src was abolished when AR was knocked down by transfection with AR siRNA (Fig. 4A). Similarly, pretreatment with nilutamide, an AR antago-

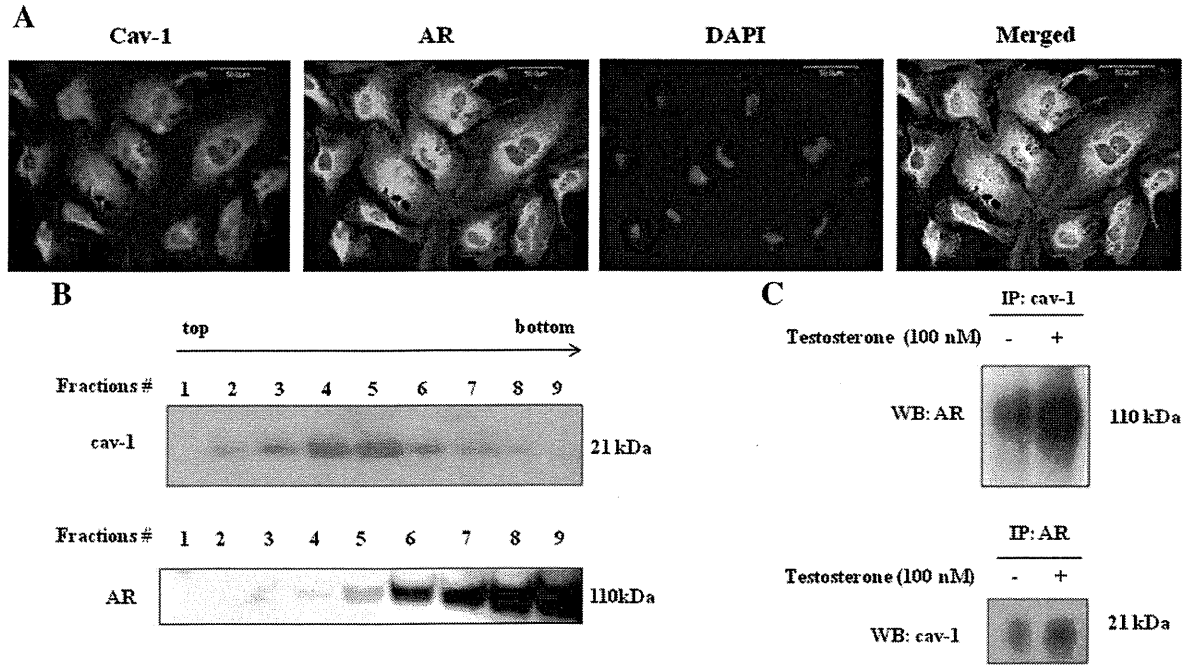

Fig. 2. AR was co-localized with caveolin-1 (cav-1) and distributed to caveolae in response to testosterone. (A) Confocal immunofluorescent images of AR and caveolin-1 in EC. Images of cells fixed on slides were merged by confocal microscopy as described in Section 2. An antibody mixture of rabbit anti-AR (N-20) and mouse anti-caveolin-1 were used to detect the proteins in ECs, followed by appropriate secondary antibodies (*red*, for caveolin-1 and *green*, for AR). Cells were incubated for 5 min with DAPI to stain nuclei. "Merged" shows an overlay of the caveolin-1 (*red*), AR (*green*) and DAPI (blue) signals. (B) Distribution of AR in caveolin-1-enriched caveolae membrane domain fraction. Cell fractionation was carried out by OptiPrep density gradient centrifugation as described in Section 2, and then developed by immunoblotting. Representative data for immunoblotting of cell fractionation from the tubs top fraction 1 to the bottom fraction 9 are shown. (C) Direct interaction of AR with caveolin-1 (cav-1). Cell lysates of ECs treated for 30 min with testosterone (100 nm) or vehicle were subjected to immunoprecipitation with antibodies against caveolin-1 or AR, separated by SDS–PAGE, detected with anti-AR or anti-caveolin-1 antibody, respectively. A representative result from three independent experiments is shown.

nist also abolished the testosterone-induced rapid phosphorylation of Src (Fig. 4B). Furthermore, immunoprecipitation assay revealed the association between AR and c-Src, which was increased by exposure of cells to testosterone for 30 min (Fig. 4C). Together with above-mentioned immunoprecipitation results about AR and caveolin-1, these data suggest that a complex formation among AR, c-Src and caveolin-1 in the plasma membrane was involved in testosterone-induced rapid eNOS activation.

4. Discussion

In the present study, we demonstrate that a caveolae-localized, AR-centered, multi-molecular complex is critical in rapid membrane-initiated eNOS activation induced by testosterone. Src kinase was shown to be an upstream regulator of the Akt and eNOS activation pathways.

Molecular, cellular and animal studies convincingly demonstrate that sex steroid hormones have various effects on vascular cells, and that many of these effects are achieved through rapid, membrane-initiated receptor-dependent signaling responses, which are different from the classical genomic actions [20]. AR is expressed in vascular ECs [17,21], and a number of reports have indicated that testosterone appears to have very rapid effects on the vascular system, including vasodilatation [22,23]. It has been shown that physiological concentrations of testosterone causes acutely (in minutes) NO-dependent vasodilatation via AR-mediated eNOS activation [24,25], which is consistent with the nongenomic nature of the response in arteries and intact ECs [26,27]. In the present study, we further demonstrated the signaling cascade driven from activation of membrane AR, which may explain mechanisms underlying rapid effects of testosterone on the vascular system.

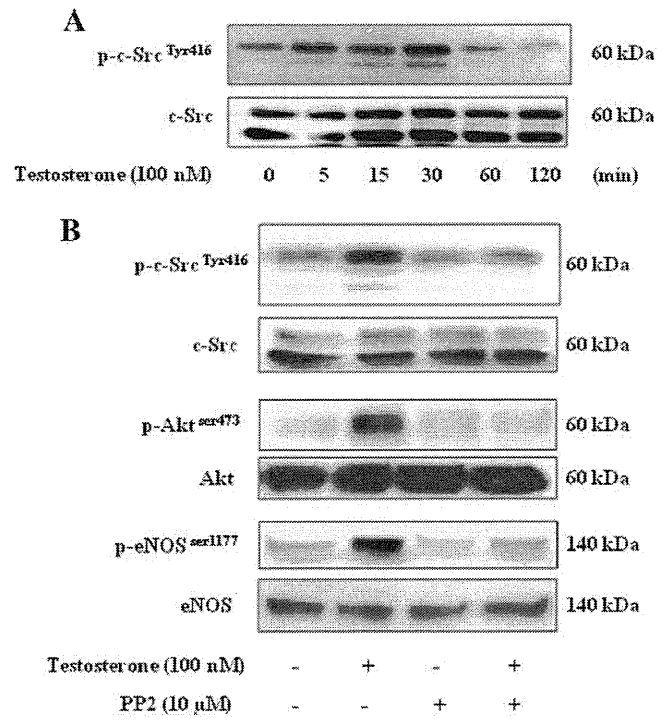

Fig. 3. Src kinase is a critical upstream regulator of Akt/eNOS activation pathway. (A) Steroid-deprived, serum-starved EC were treated with testosterone (100 nM) or vehicle for the indicated times. (B) PP2 (10 μM) was added 60 min before cells were treated with testosterone (100 nM) for 30 min. (A and B) Phosphorylation of c-Src kinase, Akt and eNOS (p-Src^{Tyr416}, p-Akt^{ser473}, p-eNOS^{ser1177}) and their total protein levels were analyzed using immunoblotting. A representative blot of three independent experiments with comparable results is show.

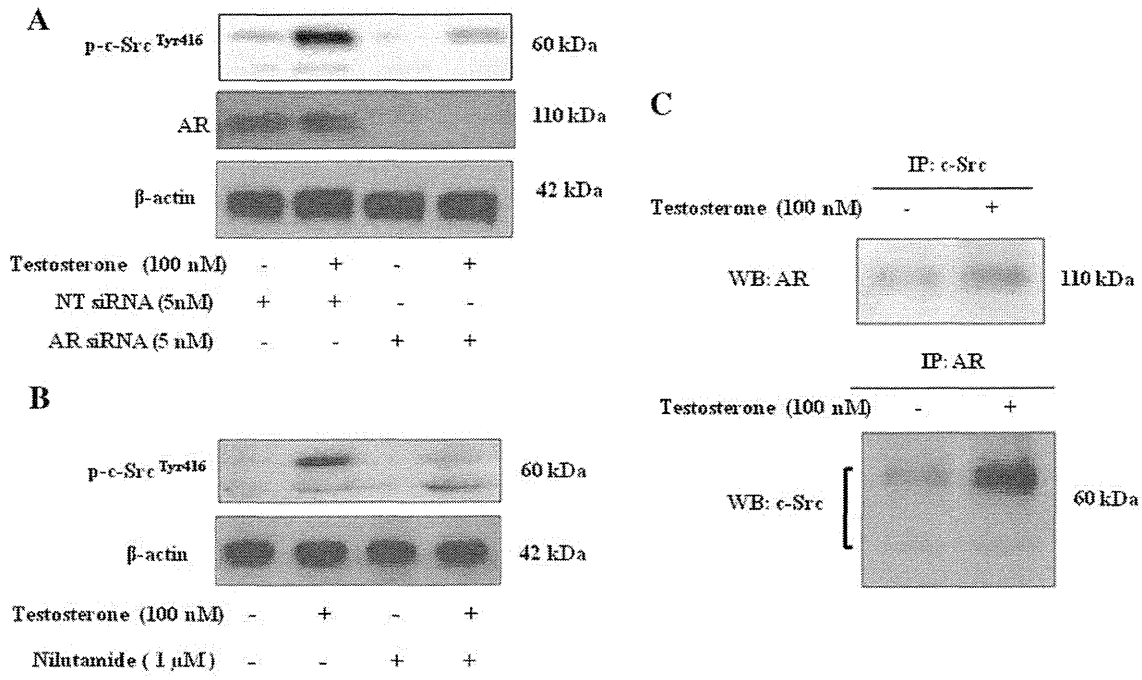

Fig. 4. The interaction between AR and c-Src. (A) Steroid-deprived, serum-starved cells were treated with testosterone or vehicle for 30 min after transfection of siRNA for AR or non-target siRNA (NT-siRNA). (B) Nilutamide (1 μM) was added 60 min before cells were treated with testosterone (100 nM) for 30 min. (A and B) Phosphorylation of c-Src kinase and AR or β-actin were analyzed using immunoblotting. A representative blot of three independent experiments with comparable results is show. (C) Cell lysates of EC treated for 30 min with testosterone (100 nM) or vehicle were subjected to immunoprecipitation with antibody against caveolin-1 or AR, separated by SDS–PAGE, detected with anti-AR or anti-caveolin-1 antibody, respectively. A representative result from three independent experiments is shown.

To further support the presence of functional membrane AR on EC, firstly we performed a series of experiments to compare the effects of the membrane-impermeable testosterone analog, testosterone-BSA with those of testosterone. Testosterone-BSA has been widely uses as a selective membrane AR ligand to study non-genomic actions of testosterone. Using testosterone-BSA and a transcriptional inhibitor actinomycin D, we showed that the rapid effect of testosterone on eNOS activation is independent of nuclear transcription activities, and that plasma membrane AR is responsible for this signaling pathway, similar to the non-genomic actions of other steroids such as estrogens [13,27,28]. In vascular ECs, caveolae are identifiable plasma membrane invaginations. Caveolin-1 is a caveolar structural protein with a long intramembrane domain, which directs caveolae targeting of multiple signaling molecules including Src family tyrosine kinases, PI3-kinase and steroid sex hormone receptors [28–31]. In the present study, confocal images and cell fractionation experiments confirmed the co-localization of AR and caveolin-1 in the plasma membrane, providing an evidence for above-mentioned non-genomic action of testosterone in EC.

The location-sensitive, membrane-associated non-receptor tyrosine kinase, Src, plays a physiological role in vascular function including vasorelaxation [13,27]. Others have indicated that Src activates PI3-kinase through steroid hormone receptors as a signaling cascade [32,33]. In the present study, c-Src interacted directly with AR in the caveolin-1-enriched membrane domain. Testosterone stimulated rapidly c-Src phosphorylation on Tyr416 which was abolished by an AR antagonist and by transfection of AR siRNA. Furthermore, a Src kinase specific inhibitor blocked the increase in phosphorylations of c-Src, Akt and eNOS. Taking these results together, we can conclude that a sequential cascade, AR-initiated c-Src/PI3-kinase/Akt activation is mediated in testosterone-induced rapid eNOS action in EC.

In summary, we demonstrated that testosterone induces the rapid assembly of a membrane-initiated signaling complex among AR, c-Src and caveolin-1, which facilitates activation of c-Src/PI3-kinase/Akt cascade with consequent activation of eNOS in vascular ECs. These findings support the concept of rapid membrane-initiated testosterone responses in the vascular endothelial system, and may provide evidence or an explanation for the favorable effects of testosterone on vascular function.

Disclosure statement

The authors have nothing to disclose.

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Videoendoscopic assessment of swallowing function to predict the future incidence of pneumonia of the elderly

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SUMMARY The purpose of the present study was to examine what dysphagic signs identified by videoendoscopy (VE) could predict the incidence of pneumonia and body weight loss in elderly patients living in nursing homes. This study was performed at six nursing care facilities in Japan from March 2007 to February 2009. The 148 subjects (85·1 ± 8·0 years, male/female: 43/105) were evaluated for their feeding and swallowing movements by clinical and VE examinations during the consumption of a regular meal. The VE examination items included the existence/absence of pharyngeal residue, laryngeal penetration, and aspiration of food and saliva. The patients were followed-up for 3 months with individualized feeding therapy based on the results of the clinical/VE examination at baseline, and the incidence of pneumonia was examined as the primary outcome. In patients without pneumonia, the body weight change was also measured as a secondary outcome. The risk factors for pneumonia and body weight loss (of 3% or more) were identified among the clinical/VE examination items by a Cox proportional hazard analysis. Even with elaborative feeding therapy, 12 (8·1%) of the 148 patients developed pneumonia during the 3 months follow-up period. The existence of signs of 'silent aspiration of saliva' or 'aspiration of saliva' detected by VE examination was a significant risk factor for both pneumonia and a body weight loss of 3% or more. This study shows that 'aspiration of saliva' detected by VE is a significant risk factor for both pneumonia and body weight loss in elderly patients living in nursing homes.

KEYWORDS: videoendoscopy, aspiration-related pneumonia, dysphagia, aspiration of saliva, body weight loss

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Introduction

Dependent elderly patients are at high risk for feeding and swallowing disorders as a consequence of disease and/or aging (1–3). Studies done in long-term care facilities have shown a prevalence of such disorders ranging from 60% to 87% (4, 5). Among the various disorders, special attention has been given to dysphagia because it may lead to malnutrition with immune system compromise, dehydration, asphyxiation, or even aspiration pneumonia (1–3). Moreover, a previous follow-up study of patients with dysphagia in such care facilities revealed an incidence of pneumonia of 43% and a mortality rate of 45% at 1 year following the detection of their swallowing disorder (6). Therefore, clinicians should be able to identify dysphagia in order to predict those patients at risk of developing complications secondary to dysphagia, as well as to develop and implement a rehabilitation plan stressing prevention and compensation.

Videofluorography (VF) has been regarded as the most popular adjunctive instrument for the

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examination of patients with suspected oropharyngeal dysphagia. Previous studies have examined the use of VF as a means to predict those at risk for dysphagia and its complications (7, 8). For instance, Mann *et al.* (7) found that the single best independent predictor for chest infection following an acute stroke was a delayed or absent swallowing response in acute stroke patients. Teraoka *et al.* (8) found that the single best predictor of oral intake in post-stroke patients with dysphagia was the presence of aspiration detected by VF assessment. Nevertheless, one major disadvantage of VF for patients living in long-term care facilities is that the patients need to be transported to a hospital setting, which is sometimes inconvenient or may disorientate the patient because of the sudden change in the environment. Other disadvantages are related to the exposure to x-ray radiation and the risk of aspiration during VF assessment in some patients with severe physical or mental alterations (9).

On the other hand, videoendoscopic (VE) examination of swallowing allows for easy assessment of patients in their usual environment because the instrument is portable and does not require a radiology suite (10). Additionally, although VE is most useful for the examination of the integrity of the upper airway before and after a swallow response, it enables the evaluation of the tongue function during mastication and deglutition, as well as the detection of aspiration by the objective visualization of the airway (11, 12).

Videoendoscopic examination has been shown to successfully estimate the existence of accumulated oropharyngeal secretions, thus resulting in excellent prediction of aspiration (13, 14). In addition, Ota *et al.* (15) reported that the secretion scale based on the VE examination is a useful evaluation tool for predicting not only aspiration, but also pneumonia, in acute-phase dysphagic stroke patients. Furthermore, Link *et al.* (16) reported that there was a relationship between the VE-based pooled hypopharyngeal secretions, laryngeal penetration, aspiration and recurrent pneumonia with neurological disorders in pediatric patients. It is therefore evident that VE is the best tool to examine pooled hypopharyngeal secretions, laryngeal penetration, and aspiration. Therefore, even though the agreement rate between the VF and VE findings on dysphagia was shown to be high (90%) (17), VE examinations are becoming increasingly popular for examining the aspiration of saliva and food at the bedside and in long-term care facilities (17, 18).

In a prospective study with acute stroke patients, Lim *et al.* (19) found a strong association between aspiration detected by VE and the development of aspiration pneumonia. However, the predictors of aspiration pneumonia in dependent elderly patients with dysphagia in long-term care facilities have not been sufficiently investigated using VE. Therefore, the purpose of this prospective cohort study was to investigate whether the dysphagic signs identified by VE were risk factors for pneumonia and body weight loss in patients living in long-term care facilities.

Materials and methods

Subjects

Six hundred and forty-seven inpatients were initially identified from six nursing care facilities in Tokyo, Japan from March 2007 to February 2009 (Fig. 1). All patients, except for 28 subjects who were tube-fed, were screened for dysphagia by a check-list given to the patient's caregiver. The screening check-list contained 11 items: pooling of food, uncomfortable feeling in the throat, previous history of asphyxiation, previous history of aspiration, previous history of pneumonia, increased phlegm production, choking on saliva, choking on food, choking after a meal, prolongation of their eating time, and insufficient intake. The 171 patients who had at least one item checked positively by the caregiver were suspected to have dysphagia and comprised the intended sample population. However, 23 patients were excluded because of cognitive failure or refusal to participate in this study. Consequently, the final study population consisted of 148 patients (male/female: 43/105) with a mean age of 85·1 ± 8·0 years and an age range from 59 to 100 years. The protocol for this study was approved by the Ethics Committee of the Nippon Dental University School of Life Dentistry at Tokyo (#08-10).

Baseline measurements and feeding therapy

At the baseline measurement, a medical doctor assessed the patients' general health condition, and none of the patients fulfilled the Mann's criteria (7) for a diagnosis of pneumonia, that is, the presence of at least three of the following signs and symptoms: fever >38 °C, productive cough with sputum, tachypnea higher than 22 breaths per minute, inspiratory crackles,

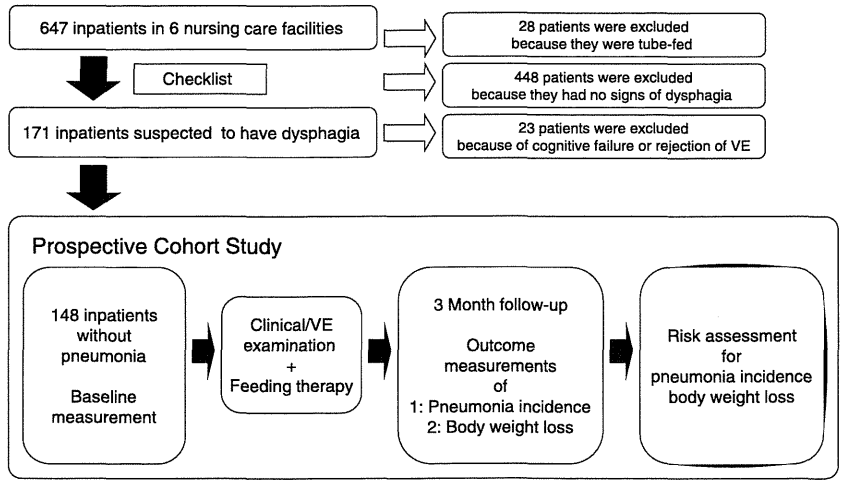

Fig. 1. The sampling process used for this study.

an abnormal chest x-ray, or positive gram staining and cultures.

All included subjects had their eating ability and dysphagic signs and symptoms evaluated clinically according to a clinical examination form regarding the signs and symptoms of dysphagia (spilling food, pooling food, oral food residue after a swallow, inability to open the mouth, choking/coughing, increased phlegm while eating, dyspnea, wet hoarseness, other), the hand and mouth coordination during the meal (feeding posture, prolongation of eating time) and the caregiver's technique used for feeding assistance.

In addition, each patient's swallowing function was examined by VE, which consisted of a flexible endoscope (ENF-V2*) connected to a high-intensity compact light source (CLH-SC*) and a video recorder (OTV-SC*).The endoscope was passed transnasally to the hypopharynx at a vantage point that provided a full view of the laryngeal vestibule, and was kept in place for a period of 10–15 min to assess the patient's eating ability, or saliva swallows when the patient was not consuming a meal. The patients were examined in their usual eating position, that is, the ambulatory patients were seating in the upright position, while the bed-bound patients were sitting on a bed. All swallows were recorded on videotapes for the further analyses by experienced physicians familiar with endoscopic swallowing studies and who were blinded to the intentions of the study. Each patient's video-recording data were reviewed for the presence or absence of pharyngeal residue, and penetration and aspiration of food or saliva. 'Penetration' was defined as a passage of material into the larynx that does not pass below the vocal folds, while 'aspiration' was defined as passage of material below the level of the vocal folds. In cases where the aspiration of food or saliva did not induce a cough, it was defined as 'silent aspiration' according to the criteria proposed by Rosenbek *et al.* (1996) (20). To assess the inter-rater reliability of the swallowing evaluations, the three investigators who were unaware of the original evaluation results, separately reviewed a random 10% sample of these evaluations. The overall agreement rate between investigators was substantial according to the Landis and Koch criteria (21) (kappa coefficient = 0·660).

On the basis of these aforementioned evaluations, the patients received various feeding therapies (22) during the follow-up period, for example, confirmation of feeding conditions [76 patients (51·4%) of 148 patients, multiple answers possible], appropriate feeding assistance [69 patients (46·6%)], food modification [32 patients (21·6%)], modification in feeding posture [19 patients (12·8%)] and modification in food intake [four patients (2·0%)] for 3 months. Food modification involved changing the dietary consistency. We modified the food and liquid texture individually according to the National Dysphagia Diet recommendations (23). Food intake and feeding assistance required modifications to accommodate the individual needs of the patients, such as changes in the rate and amount of the food consumed, appropriate utensils and the

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method used for self-feeding (22). Modifications in the feeding posture were applied in order to maximize the physical capabilities and improve swallowing, and involved strategies such as head-turn or chin-tuck maneuvers or whole body-positioning strategies including the patient tilting to the side or back, side-lying, or maintaining an upright posture (22). All patients received oral health care after every meal by the caregiver who was instructed once a week about the oral care procedures by a dental hygienist. Caregivers cleaned each patient's oral cavity using a toothbrush for approximately 5 min after each meal. The brushing was carried out as usual for daily tooth brushing without paste, and included brushing the palatal and mandibular mucosa and tongue dorsum. Dentures were also cleaned with a denture brush every day.

The 3 month follow-up and outcome measurement

The first outcome variable after 3 months of follow-up was the incidence of pneumonia diagnosed according to the same criteria applied at the baseline measurement. Once the patients received a diagnosis of pneumonia, they were sent to a local hospital for treatment, without exception. Consequently, their oral feeding was prohibited to prevent further aspiration pneumonia and their body weight typically decreased as a result (24). The incidence of pneumonia and body weight loss were therefore strongly correlated after the development of pneumonia. Thus, when pneumonia was identified, follow-up measurements of the patient's body weight were terminated.

The second outcome variable during the follow-up period was a change in body weight demonstrated by monthly measurements. Since there is a close relationship between pneumonia and body weight loss, the incidence of body weight loss of 3% or more was examined in patients who had not been diagnosed with pneumonia during the 3 months of follow-up. Once the patients developed a body weight loss of 3% or more, the patients received some form of nutrition therapy, and thus, the follow-up observation was terminated.

Statistical analysis

A survival curve of the patients who had not been diagnosed with pneumonia was drawn for a Kaplan–Meier analysis. According to the presence/absence of pneumonia during the 3 months of follow-up, we divided the final sample population into pneumonia and non-pneumonia sub-groups, and performed a *t*-test, chi-square analysis or Fisher's exact test to analyse the differences between the two groups.

Similarly, a survival curve of those patients who had not lost more than 3% of their body weight was drawn for a Kaplan–Meier analysis (outcome event: the incidence of body weight loss of 3% or more). Differences between the weight gain/no change sub-group (body weight gain, or a small weight loss of no more than 3% of the initial body weight) and the weight loss group (body weight loss of 3% or more (10, 25)) were analysed with the same statistical tests utilized for the incidence of pneumonia.

Additionally, a Cox proportional hazard analysis was performed to identify the risk factors for the incidence of pneumonia and the body weight loss of 3% or more. The analysed predictors were age, self-feeding ability, the Barthel activities of daily living (ADL) index, a body mass index (BMI) lower than 18·5, pharyngeal residue, laryngeal penetration, aspiration of food and aspiration of saliva. Regarding the aspiration of food or saliva, the data were handled as ordinal variables (negative, positive, positive as silent aspiration). The data were analyzed with the Statistical Package for the Social Sciences software program (SPSS version 15.0[†]). A *P*-value <0·05 was considered to be statistically significant.

Results

Baseline condition of the patients

Examination of the medical conditions of the initial 148 patients showed the presence of a prior stroke in 83 (comorbidity admitted) (56·1%), dementia in 74 (50·0%), Parkinson's disease in 10 (6·8%), cardiovascular disease in 10 (6·8%), hypertension in 8 (5·4%), previous pneumonia in 5 (3·4%), diabetes mellitus in 3 (2·0%), fractures in 3 (2·0%) and other comorbidities in 14 patients (9·5%).

The clinical examination regarding the eating ability and signs and symptoms of dysphagia before the VE evaluation showed choking/coughing in 110 out of 148 patients (multiple choice admitted), pooling of food in 28, prolongation of the eating time in nine, inability to

[†]SPSS Japan Inc., Tokyo, Japan.

open the mouth in two, and spilling of food in one patient.

The VE evaluation detected pharyngeal residue in 97 (65·5%) out of the 148 patients, laryngeal penetration in 67 (45·3%), aspiration of food in 41 (27·7%), silent aspiration of food in 19 (12·8%), aspiration of saliva in 8 (5·41%), and silent aspiration of saliva in 10 (6·76%) patients (Table 1).

Risk factors for pneumonia and body weight loss

Even with elaborative feeding therapy, during the 3 months of follow-up after the baseline measurement, 12 (8·1%) of the 148 patients developed pneumonia (Fig. 2). In addition, among the non-pneumonia patients, 90 (66·2%) of them presented with weight gain, no change or weight loss of 3% or less (weight gain/no change group), while 46 patients (33·8%) lost 3% or more of their body weight (weight loss group) (Fig. 3).

The differences between the pneumonia and non-pneumonia groups concerning the clinical/demographic data and the dysphagic signs detected by VE are shown in Table 1. The unpaired *t*-test showed that there were no significant differences in the patient age ($P = 0·505$), gender ($P = 0·244$), self-feeding ability ($P = 0·419$), number of patients with a BMI lower than 18·5 ($P = 0·190$), and the Barthel Index ($P = 0·060$) between the subjects with and without pneumonia. On the other hand, there was a significant difference in the frequency of 'aspiration of saliva' between the pneumonia and non-pneumonia patients ($P = 0·026$). In contrast, a comparison between the body weight gain/no change and body weight loss groups showed that there were no significant differences concerning any of the analysed variables (Table 2).

The results of the Cox proportional hazard analysis revealed that a sign of the 'aspiration of saliva' detected by VE was a significant risk factor for pneumonia (Table 3) and for a body weight loss of 3% or more (Table 4).

Discussions

The presence of aspiration-related pneumonia is known to be associated with a high mortality rate in the elderly. Patients in nursing homes may have a higher incidence of pneumonia because of their multiple underlying diseases, which may lead to immunosuppression, excessive use of medications, generalized decreased functional status, as well as factors related to malfunctioning of the masticatory and orophraryngeal systems and inadequate oral care. In particular, dysphagia is known to be strongly associated with aspiration pneumonia. Teramoto *et al.* (26). reported

Table 1. The relationship between the clinical/VE signs and the incidence of pneumonia

	Total subjects	No pneumonia ($n = 136$)	Pneumonia ($n = 12$)	*P*-value
Age (mean ± s.d.)	148	85·0 ± 8·1	86·8 ± 5·4	0·505†
Male/female	148	38/98	5/7	0·244††
Self-feeding (yes/no)	148	47/89	5/7	0·419††
Barthel Index (mean ± s.d.)	116*	13·1 ± 18·1	7·2 ± 7·12	0·060†
BMI < 18·5**	118**	43/110 (39·1%)	5/8 (62·5%)	0·190††
Pharyngeal residue	148	88 (64·7%)	9 (75·0%)	0·354††
Laryngeal penetration	148	62 (45·6%)	5 (41·7%)	0·519††
Aspiration of food	148			0·326††
Silent aspiration	19	19	0	
Aspiration	41	38	3	
NA	88	79	9	
Aspiration of saliva	148			0·026††
Silent aspiration	10	7	3	
Aspiration	8	7	1	
NA	130	122	8	

*Of 116 patients, 107 were in the no pneumonia group and nine were in the pneumonia group.
**Of 118 patients, 110 were in the no pneumonia group and eight were in the pneumonia group.
†*T*-test.
††Chi-square test.

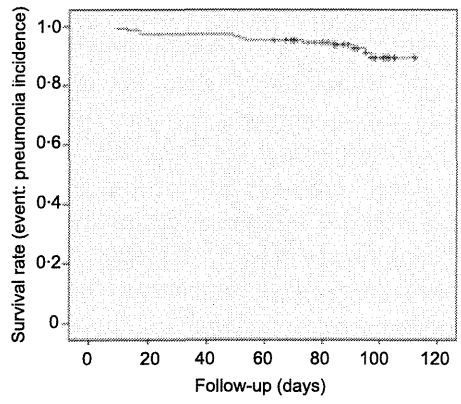

Fig. 2. The survival curve of the patients who did not suffer from pneumonia. The survival curve was drawn for a Kaplan–Meier analysis (outcome event: incidence of pneumonia).

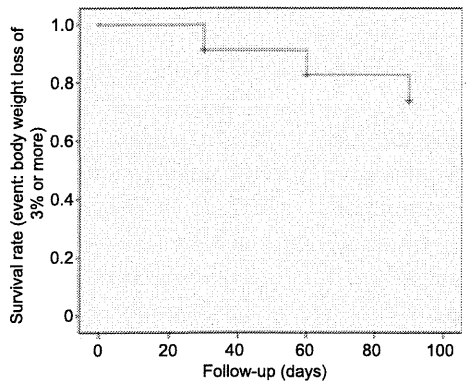

Fig. 3. The survival curve of the patients who did not suffer from a body weight loss of 3% or more. The survival curve was drawn for a Kaplan–Meier analysis (outcome event: incidence of body weight loss of 3% or more).

that 70% of the pneumonia in the elderly occurred due to aspiration, and Yamaya *et al.* (27). reported a high prevalence of silent aspiration in older persons leading to the deterioration of swallowing function due to cerebrovascular disease. In a previous study, Doggett *et al.* (28) estimated that approximately 43–54% of stroke patients have dysphagia and aspiration of food or saliva, and that approximately 37% of these patients would develop aspiration-related pneumonia.

In this present study, penetration and aspiration (apparent or silent) was observed in 67 subjects (45·2%) and 60 subjects (40·5%), respectively. The prevalence of aspiration found in this investigation was relatively high compared to previous studies utilizing VE examination (29%) (29), but was similar to the range observed in a previous review article where it was reported to occur in 15–39% of subacute dysphagic stroke patients (30). According to this review, the exact prevalence of aspiration remains unknown because of the differences in the size and methodology used in the existing studies.

The incidence of pneumonia was 12 (8·1%) among the 148 subjects (Table 1), which is in accordance with the study by Lim *et al.* (19), who reported that five patients (10%) developed pneumonia during their inpatient stay, and that all of them were at risk of aspiration of saliva or food as determined by a VE examination. On the other hand, Croghan *et al.* (6) reported that 55% of their nursing home patients presented with aspiration on VF examination, and 43% developed pneumonia.

One possible reason for such a discrepancy in the association of pneumonia and aspiration or penetration could be due to the technique (VE vs. VF) utilized to assess the swallowing disorders. Although a number of methods have been used to detect the symptoms of dysphagia, it is very difficult to evaluate 'silent aspiration of saliva' with a bedside clinical assessment alone, because it has been shown that it is missed in up to 40% of the patients aspirating silently (31, 32). At present, VF and VE are regarded as the best methods to evaluate swallowing function. In particular, VF has been used as a gold standard to evaluate swallowing because it can detect aspiration. However, it may not be as accurate in identifying 'silent aspiration of saliva', as compared to VE, because the latter enables direct visualization of the aspiration of saliva (18, 33, 34). Kelly *et al.* (35) reported that penetration and aspiration are perceived more sensitively in VE images than in VF images of the same swallows. It is also well known that VE can identify the microaspiration and aspiration of secretions with a high reliability, whereas VF cannot (36, 37). Additional advantages of VE are related to its application. Inpatients may become agitated or fatigued in the radiology suite or may not respond well to the taste of barium-coated boluses, or may even reject the radiation exposure, limiting the applications of VF. Videoendoscopy allows the patient's examination to be performed regardless of his/her altered mental status or immobility (38). Finally, Wu *et al.* (39) stated that VE is conclusively a safe, more efficient and sensitive method than VF for evaluating swallowing.

Another reason for the discrepancy could be the effect of the feeding therapy provided in this study, which could have reduced the symptoms of dysphagia,

Table 2. The relationship between the clinical/VE signs and the change in body weight

	Total subjects	Gain/no change (n = 90)	Weight loss (n = 46)	P-value
Age (mean ± s.d.)	136	84·6 ± 8·0	85·7 ± 8·6	0·464†
Male/female	136	25/65	13/33	0·553††
Self-feeding (yes/no)	136	29/61	16/30	0·454††
Barthel Index (mean ± s.d.)	107*	14·9 ± 18·7	9·6 ± 17·0	0·163†
BMI < 18·5	110**	30/74 (40·5%)	13/36 (36·1%)	0·655††
Pharyngeal residue	136	61 (67·8%)	27 (58·7%)	0·294††
Laryngeal penetration	136	44 (48·9%)	18 (39·1%)	0·2797††
Aspiration of food	136			0·975††
Silent aspiration	19	13	6	
Aspiration	38	25	13	
No aspiration	79	52	27	
Aspiration of saliva	136			0·342††
Silent aspiration	7	4	3	
Aspiration	7	3	4	
No aspiration	122	83	39	

Weight loss was diagnosed as the loss of 3% or more of the body weight from the baseline measurement.
*Of the 107 patients, 72 were in the gain/no change group and 35 were in the weight loss group.
**Of the 110 patients, 74 were in the gain/no change group and 36 were in the weight loss group.
†T-test.
††Chi-square test.

Table 3. The results of the Cox proportional hazard analysis for the possible predictors of the incidence of pneumonia

Predictors	B	P-value	HR	95% CI
Age	0·011	0·860	1·011	0·900–1·135
Self-feeding	0·105	0·909	1·111	0·182–6·785
Barthel Index	−0·010	0·769	0·990	0·927–1·057
BMI < 18·5	2·064	0·070	7·874	0·844–73·440
Pharyngeal residue	−0·621	0·615	0·537	0·048–6·067
Laryngeal penetration	0·571	0·642	1·771	0·160–19·644
Aspiration of food (negative/positive/positive with SA)	−0·216	0·830	0·805	0·112–5·794
Aspiration of saliva (negative/positive/positive with SA)	1·290	0·025	3·634	1·174–11·242

HR, hazard ratio; CI, confidence interval; SA, silent aspiration.

Table 4. The results of the Cox proportional hazard analysis for the possible predictors of a body weight loss of 3% or more

Predictors	B	P-value	HR	95% CI
Age	0·019	0·448	1·019	0·971–1·070
Self-feeding	0·530	0·228	1·698	0·718–4·014
Barthel Index	0·000	0·992	1·000	0·976–1·025
BMI < 18·5	0·859	0·032	2·362	1·074–5·191
Pharyngeal residue	−0·060	0·896	0·942	0·381–2·325
Laryngeal penetration	0·019	0·970	1·019	0·374–2·780
Aspiration of food (negative/positive/positive with SA)	−0·203	0·569	0·816	0·405–1·644
Aspiration of saliva (negative/positive/positive with SA)	1·186	0·000	3·275	1·828–5·866

HR, hazard ratio; CI, confidence interval; SA, silent aspiration.

pharyngeal residue, laryngeal penetration, and aspiration of food, as demonstrated by the fact that 66% of the subjects were able to increase their body weight or keep the body weight loss to within 3%. Nevertheless, a detailed analysis of the effectiveness of feeding therapy on the reduction of the symptoms of dysphagia could not be performed, because it was beyond the scope of this study.

Additionally, the differences in the target populations and their respective medical conditions could also have affected the overall incidence of pneumonia. This study gathered a heterogeneous patient population consisting of patients presenting with well-known disorders/diseases associated with the symptoms of dysphagia (e.g. stroke, Parkinson's disease, dementia) as well as other non-debilitating diseases/disorders (hypertension, fractures). On the other hand, a strong point in this study was the inclusion of a relatively high number of subjects from six nursing care facilities, which was large compared to other follow-up studies. Therefore,

the incidence of pneumonia may have been relatively lower in such a large heterogeneous study sample.

Regarding the risk factors associated with the development of pneumonia, some of them were reported to be age, primary disease, consciousness disorders, nutritional status, poor ADL, poor oral status, and swallowing dysfunction (40, 41). In the present study, among the analysed predictors, the 'aspiration of saliva' detected by VE was the only significant risk factor for pneumonia. In cases of bad oral health, saliva contains numerous bacteria. Therefore, patients with silent aspiration of saliva (without a cough reflex) are aspirating bacteria, which may be the main factor responsible for increasing the risk of pneumonia.

Additionally, even with the elaborative feeding therapy provided in this study, the control of aspiration of saliva or silent aspiration of saliva was generally difficult. In the present study, there was also a tendency for there to be a higher incidence of pneumonia in poor ADL patients. Langmore *et al.* (42) also reported that severely dependent functional status was an especially potent predictor of aspiration pneumonia. Riquelme *et al.* (40) reported that there was a significant relationship between the ADL and mortality rate. It was also observed that patients with a BMI < 18·5 had a higher tendency to develop pneumonia ($P = 0{\cdot}070$) compared with those with a poor ADL ($P = 0{\cdot}769$). It is well known that a lower nutrition condition affects the host immunological function, thus making the subjects more susceptible to pneumonia (43).

On the other hand, aspiration of saliva was also detected as a significant risk factor for body weight loss in this study. This finding could be explained by the possible presence of subclinical aspiration-related pneumonia in those subjects with a body weight loss of 3% or more.

The overall findings in this study demonstrated that it is still very difficult to prevent aspiration of saliva even if physicians provide elaborative feeding therapy and even if patients do not eat and drink anything through the mouth. Effective strategies to prevent the silent aspiration of saliva will therefore be an important target for future research.

Conclusion

The results of this study showed that, even with elaborative feeding therapy, 'aspiration of saliva' as detected by videoendoscopic examination was found to be a significant risk factor for pneumonia and a body weight loss of 3% or more in elderly patients living in nursing homes.

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