tients should not be treated with chemotherapy because there is no tumor cell and chemotherapy will only do harm (14). Therefore, it is very important to recognize the type of amyloidosis by performing immunohistochemical staining.

Congenital heart disease

Atrial septal defect (ASD) is the most common congenital lesion in adults. While the patients with an ASD are often asymptomatic until adulthood, most patients with significant shunt flow will become symptomatic by the age of 40 (15). Right ventricular failure can be the presenting symptom in older patients. Atrial arrhythmias increases with age and precipitates HF (16, 17). In one study, for example, the incidence of atrial fibrillation or atrial flutter prior to surgery was 1 percent for those aged 18 to 40, 30 percent for those aged 40 to 60, and 80 percent in those over the age of 60 (16).

Precipitating Factors of HF in the Elderly

Exacerbation of HF in patients with underlying heart disease is often accompanied by some of the following precipitating factors. Identification of these precipitating factors of HF is very important because an episode of HF can often be terminated by treating a specific precipitating factor and may even be prevented by avoiding them.

Arrhythmia

Both tachyarrhythmia and bradyarrhythmia can precipitate HF.

The most common tachyarrhythmia in elderly population is atrial fibrillation (AF). The prevalence of AF was 9 percent in those 80 years of age compared to 0.1 percent among adults younger than 55 years of age (18). Thus, AF is primarily a disease of older subjects. Loss of atrial booster pump function with AF impairs ventricular filling and loweres cardiac output. This loss of atrial contraction is particularly deleterious in elderly patients with a less compliant left ventricle. In addition, uncontrolled rapid ventricular responses with AF reduce the time available for ventricular filling. This exacerbates diastolic dysfunction and raises atrial pressure, thus precipitating HF.

The incidence of bradyarrhythmia, such as sick sinus syndrome and AV block, also increases with age. This is probably due to age-associated fibrosis and sclerosis of the conduction system. Marked bradycardia in a patient with underlying heart disease depresses cardiac output, because stroke volume may already be maximum and cannot rise farther to maintain cardiac output.

Renal failure

Renal function deteriorates as people get old. Recent studies showed that renal dysfunction is an independent and important predictor of all-cause mortality in patients with HF (19, 20). As renal failure impairs the ability of patients with heart failure to excrete sodium and fluid, volume overload will develop and thus exacerbate HF symptoms. Therefore, renal failure is an important precipitating factor of HF in the elderly.

Anemia

Anemia is often encountered in elderly patients with HF. Chronic anemia is associated with high cardiac output when hemoglobin is less than 8 gm/dl. Decreased blood viscosity and a reduced arteriolar tone resulted from tissue hypoxia contribute to reduced systemic vascular resistance which cause an high-output status. Development of high-cardiac output status in the presence of underlying heart disease often precipitates HF because preload reserve is already limited and left ventricular end-diastolic pressure will increase. Anemia also can exacerbate myocardial dysfunction in the presence of coronary heart disease due to myocardial ischemia caused by reduced oxygen delivery. Severe anemia in patients with HF should be corrected.

Infection

Systemic infection can precipitate HF. The mechanism responsible for worsening HF is increased total body oxygen consumption due to fever. Patients with HF are particularly susceptible to respiratory tract infection, presumably because of diminished ability of congested lungs to expel respiratory secretions.

Pulmonary embolism

Elderly patients with HF are at high risk of developing pulmonary embolism, particularly when confined to bed. Pulmonary embolism can exacerbate HF by increasing the hemodynamic burden on the right ventricle and worsening hypoxemia.

Non-compliance with medication and/or diet

A common cause of decompensation in a previously compensated elderly patient with HF is non-compliance with medication and/or diet (21). Inappropriate reduction of therapy, in forms of dietary sodium and fluid restriction or pharmacological therapy, precipitate HF. Nowadays patients with HF take a complex medication regimen and have difficulties in adhering to it. Education of the patient and family is necessary to prevent this problem.

Adverse effect of drugs

Drugs with negative inotropic action such as beta blockers and calcium antagonists can aggravate HF. More importantly, non-steroidal anti-inflammatory drugs (NSAIDs) exacerbate preexisting HF. They are often prescribed for the elderly because of arthralgia. In a report of elderly patients with heart disease, there was a 10-fold increased risk of exacerbating HF of sufficient severity to require hospitalization in patients with recent NSAIDs use, compared to those without such use; the risk was related to the dose of NSAIDs consumed within the week prior to hospitalization (22). NSAIDs use blunts the renal effects of diuretics among pa-

Table 1. Mean Age of Patients in Large Heart Failure Trials

Trial	Drug	Mean age	Exclusion criteria
V-HeFT I	hydralazine, ISDN	58	<75
V-HeFT II	enalapril	61	<75
CONSENSUS	enalapril	70	none
SOLVED-Treatment	enalapril	61	<80
ELITE-II	losartan	71	60>
Val-HeFT	valsartan	63	none
DIG	digoxin	63	none
US carvedilol trial	carvedilol	58	none
MERIT-HF	metoprolol CR	64	<80
CIBIS II	bisoprolol	61	<80
COPERNICUS	carvedilol	63	none
RALES	spironolactone	65	none

tients with HF, thereby resulting in an enhanced risk of exacerbating HF. One study of over 10,000 elderly patients found that the concurrent use of NSAIDs and diuretics was associated with a two-fold increased risk of hospitalization for HF, compared to the use of diuretics alone (23).

Diagnosis of HF in the Elderly

Diagnosis of HF may be difficult in the elderly. Symptoms of HF are often atypical or even absent in the elderly. Many older patients may not have dyspnea on exertion because of their sedentary lifestyle. Nonspecific complaints of generalized weakness, anorexia and fatigue often predominate. Some studies have reported that HF is the most frequent precipitating cause of delirium in older patients (24).

On the other hand, older patients with typical symptoms of HF are misdiagnosed with other diseases. For example, a dry cough or mild shortness of breath may be mistakenly attributed to chronic pulmonary disease. Easy fatiguability and generalized weakness may be wrongly thought merely to reflect changes associated with aging.

Given the difficulties in making a diagnosis of HF in the elderly, the possibility of using a blood test to make a diagnosis of HF is appealing. Elevated plasma levels of brain natriuretic peptide (BNP) has been shown to be a reliable indicator of HF or LV dysfunction (25). Maisel et al showed that BNP value of >100 pg/ ml was useful in establishing or excluding the diagnosis of HF in patients with acute dyspnea (26). However, this may not apply to the elderly population. In recent population-based cohort study, Redfield et al reported that BNP increased significantly with age, especially in women (27). For example, the 95th percentile range of BNP in healthy women aged >75 was 155 pg/ml in that study. Diagnosis of HF based on the BNP value needs caution in the elderly.

Treatment of HF in the Elderly

Principles of treatment of HF in the elderly is the same as those in younger patients. First of all, identification of the underlying etiology and precipitating factors of HF is essential. Then, treatment aimed at retarding or reversing the underlying conditions that predispose to the development of HF should be employed to the degree possible.

Several large-scale, randomized clinical trials have shown that various class of medications reduce the risk of death in patients with HF. Mean age of patients enrolled in these large HF trials is listed on Table 1. Patients enrolled in those trials are much younger than patients with HF in the real world. In addition, patients over 80 years of age are poorly represented in these trials (28). Although these drugs are increasingly being used in older patients with HF, the efficacy of therapies remains uncertain in the very old. Future clinical trials should adequately include the elderly population that carry the burden of the disease.

Systolic heart failure is usually managed by diuretics, angiotensin-converting enzyme inhibitors, digitalis and beta-blockers. Japanese Circulation Society issued a guideline for the treatment of HF (29). Recommendation of therapy for HF according to the severity of symptoms is shown in Fig. 3. The management of diastolic heart failure is discussed later.

When using these drugs, it is important for physicians to take into consideration that the therapeutic range is narrow in treating elderly patients with HF. Elderly patients may have diminished responses to these medications compared with younger patients and may experience a higher risk of adverse effects attributable to treatment (30, 31).

As for the non-pharmacological aspects of management of HF, some multidisciplinary HF programs have been successful in decreasing the rate of rehospitalization and associated morbidity in elderly patients (32).

Problem of Diastolic HF in the Elderly (33)

Approximately 20 to 40 percent of patients with HF have preserved left ventricular systolic function and are believed to have impaired ventricular relaxation as the primary

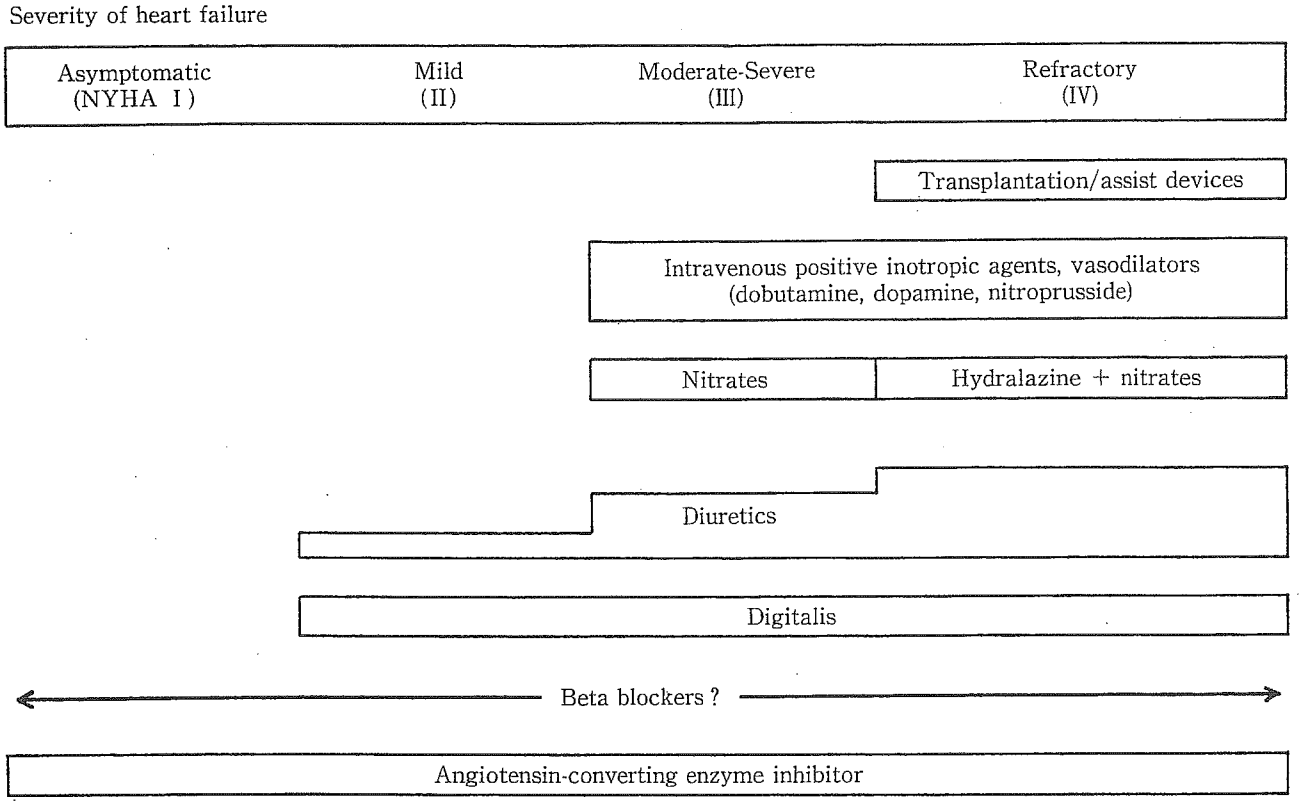

Figure 3. Therapy for heart failure in relation to the severity of symptoms (29).

mechanism of HF (34). Heart failure associated with preserved systolic function is primarily a disease of elderly, most of whom have hypertension (35). This observation may be related to the fact that aging has a greater impact on diastolic function than on systolic performance (36). Aging is associated with decreases in the elastic properties of the heart and great vessels, which leads to an increase in systolic blood pressure and an increase in myocardial stiffness. The rate of ventricular filling decreases in part because of structural changes in the heart (due to fibrosis) and because of a decline in active relaxation (due to an increase in afterload). These deleterious effects on diastolic function are exacerbated by a decrease in beta-adrenergic receptor density and a decline in peripheral vasodilator capacity, both of which are characteristic of elderly patients. In addition, elderly patients commonly have associated disorders (such as coronary artery disease, diabetes mellitus, aortic stenosis, atrial fibrillation), which can adversely affect the diastolic properties of the heart or decrease the time available for ventricular filling.

In contrast to the treatment of HF due to systolic dysfunction, few clinical trials are available to guide the management of patients with HF due to diastolic dysfunction. In the absence of controlled clinical trials, the management of patients with diastolic dysfunction is frequently determined by a set of therapeutic principles (37). These include control of hypertension, control of tachycardia, reduction in central blood volume, and alleviation of myocardial ischemia.

Hypertension exerts a deleterious effect on diastolic function by causing both structural and functional changes in the heart. Increases in systolic blood pressure have been shown to slow myocardial relaxation (38), and the resulting hypertrophy may adversely affect passive chamber stiffness. Physicians should make every effort to control both systolic and diastolic hypertension with effective antihypertensive therapy.

Tachycardia can shorten the time available for ventricular filling and coronary perfusion. Drugs that slow the heart rate or the ventricular response to atrial arrhythmias (eg, beta-blockers) can provide symptomatic relief in patients with diastolic dysfunction.

Circulating blood volume is a major determinant of ventricular filling pressure. So the use of diuretics may improve breathlessness in patients with diastolic as well as systolic dysfunction.

Because myocardial ischemia can impair ventricular relaxation, coronary revascularization should be considered in patients with coronary artery disease in whom symptomatic or demonstrable myocardial ischemia is believed to be exerting a deleterious effect on diastolic function.

Summary

HF is common in elderly population. The common causes of HF in the elderly are ischemic heart disease, valvular heart disease, hypertensive heart disease, and cardiomyopathy. Exacerbation of HF is often accompanied by precipitating factors in the elderly. Making a diagnosis of HF may be difficult in the elderly because symptoms of HF are often atypical. Heart failure with preserved systolic function is common in the elderly. Basic principles of HF treatment in the elderly is similar to those in the young patients.

References

1) Kupari M, Lindroos M, Iivanainen AM, et al. Congestive heart failure in old age: prevalence, mechanisms and 4-year prognosis in the Helsinki Ageing Study. J Intern Med **241**: 387–394, 1997.
2) Kannel WB, Belanger AJ. Epidemiology of heart failure. Am Heart J **121**: 951–957, 1991.
3) Ho KK, Pinsky JL, Kannel WB, et al. The epidemiology of heart failure: the Framingham Study. J Am Coll Cardiol **22**: 6A–13A, 1993.
4) Tsuchihashi M, Tsutsui H, Kodama K, et al. Clinical characteristics and prognosis of hospitalized patients with congestive heart failure—A study in Fukuoka, Japan—. Jpn Circ J **64**: 953–959, 2000.
5) Reynen K, Bachmann K. Coronary arteriography in elderly patients: Risk, therapeutic consequences and long-term follow-up. Coron Artery Dis **8**: 657–666, 1997.
6) Sigurdsson E, Thorgeirsson G, Sigvaldason H, et al. Unrecognized myocardial infarction: epidemiology, clinical characteristics, and the prognostic role of angina pectoris. The Reykjavik Study. Ann Intern Med **122**: 96–102, 1995.
7) Passik CS, Ackermann DM, Pluth JR, Edwards WD. Temporal changes in the causes of aortic stenosis: A surgical pathologic study of 646 cases. Mayo Clin Proc **62**: 119–123, 1987.
8) Iga K, Konishi T, Matsumura T, et al. Markedly enlarged right atrium associated with physical signs of tricuspid regurgitation: A cause of congestive heart failure in the elderly. Jpn Circ J **58**: 683–688, 1994.
9) Zhou X, Otsuji Y, Yoshifuku S, et al. Impact of atrial fibrillation on tricuspid and mitral annular dilatation and valvular regurgitation. Circ J **66**: 913–916, 2002.
10) Niimura H, Bachinski LL, Sangwatanaroj S, et al. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. N Engl J Med **338**: 1248–1257, 1998.
11) Maron BJ, Niimura H, Casey SA, et al. Development of left ventricular hypertrophy in adults in hypertrophic cardiomyopathy caused by cardiac myosin-binding protein C gene mutations. J Am Coll Cardiol **38**: 315–321, 2001.
12) Doi YL, Kitaoka H, Hitomi N, et al. Clinical expression in patients with hypertrophic cardiomyopathy caused by cardiac myosin-binding protein C gene mutation. Circulation **100**: 448–449, 1999.
13) Falk RH, Comenzo RL, Skinner M. The systemic amyloidoses. N Engl J Med **337**: 898–909, 1997.
14) Kyle RA, Spittell PC, Gertz MA, et al. The premortem recognition of systemic senile amyloidosis with cardiac involvement. Am J Med **101**: 395–400, 1996.
15) John Sutton MG, Tajik AJ, McGoon DC. Atrial septal defect in patients ages 60 years or older: operative results and long-term postoperative follow-up. Circulation **64**: 402–409, 1981.
16) Berger F, Vogel M, Kramer A, et al. Incidence of atrial flutter/fibrillation in adults with atrial septal defect before and after surgery. Ann Thorac Surg **68**: 75–78, 1999.
17) Gatzoulis MA, Freeman MA, Siu SC, et al. Atrial arrhythmia after surgical closure of atrial septal defects in adults. N Engl J Med **340**: 839–846, 1999.
18) Go AS, Hylek EM, Phillips KA, et al. Prevalence of diagnosed atrial fibrillation in adults: National implications for rhythm management and stroke prevention: the anticoagulation and risk factors in atrial fibrillation (ATRIA) Study. JAMA **285**: 2370–2375, 2001.
19) Mahon NG, Blackstone EH, Francis GS, et al. The prognostic value of estimated creatinine clearance alongside functional capacity in ambulatory patients with chronic congestive heart failure. J Am Coll Cardiol **40**: 1106–1113, 2002.
20) Dries DL, Exner DV, Domanski MJ, et al. The prognostic implications of renal insufficiency in asymptomatic and symptomatic patients with left ventricular systolic dysfunction. J Am Coll Cardiol **35**: 681–689, 2000.
21) Ghali JK, Kadakia S, Cooper R, et al. Precipitating factors leading to decompensation of heart failure. Traits among urban blacks. Arch Intern Med **148**: 2013–2016, 1988.
22) Heerdink ER, Leufkens HG, Herings RM, et al. NSAIDs associated with increased risk of congestive heart failure in elderly patients taking diuretics. Arch Intern Med **158**: 1108–1112, 1998.
23) Page J, Henry D. Consumption of NSAIDs and the development of congestive heart failure in elderly patients: an underrecognized public health problem. Arch Intern Med **160**: 777–784, 2000.
24) Rockwood K. Acute confusion in elderly medical patients. J Am Geriatr Soc **37**: 150–154, 1989.
25) McDonagh TA, Robb SD, Murdoch DR, et al. Biochemical detection of left-ventricular systolic dysfunction. Lancet **351**: 9–13, 1998.
26) Maisel AS, Krishnaswamy P, Nowak RM, et al. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med **347**: 161–167, 2002.
27) Redfield MM, Rodeheffer RJ, Jacobsen SJ, Mahoney DW, Bailey KR, Burnett JC. Plasma brain natriuretic peptide concentration: impact of age and gender. J Am Coll Cardiol **40**: 976–982, 2002.
28) Heiat A, Gross CP, Krumholz HM. Representation of the elderly, women, and minorities in heart failure clinical trials. Arch Intern Med **162**: 1682–1688, 2002.
29) Matsuzaki M, Sasayama S, Aizawa Y, et al. Japanese Circulation Society guidelines for the management of chronic heart failure. Jpn Circ J **64** Suppl. IV: 1023–1079, 2000 (in Japanese).
30) ACE Inhibitor Myocardial Infarction Collaborative Group. Indications for ACE inhibitors in the early treatment of acute myocardial infarction: systematic overview of individual data from 100,000 patients in randomized trials. Circulation **97**: 2202–2212, 1998.
31) Stafford RS, Saglam D, Blumenthal D. National patterns of angiotensin-converting enzyme inhibitor use in congestive heart failure. Arch Intern Med **157**: 2460–2464, 1997.
32) Rich MW, Beckham V, Wittenberg C, et al. A multidisciplinary intervention to prevent the readmission of elderly patients with congestive heart failure. N Engl J Med **333**: 1190–1195, 1995.
33) Hunt SA, Baker DW, Chin MH, et al. ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: Executive summary a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation **104**: 2996–3007, 2001.
34) Davie AP, Francis CM, Caruana L, et al. The prevalence of left ventricular diastolic filling abnormalities in patients with suspected heart failure. Eur Heart J **18**: 981–984, 1997.
35) Topol EJ, Traill TA, Fortuin NJ. Hypertensive hypertrophic cardiomyopathy of the elderly. N Engl J Med **312**: 277–283, 1985.
36) Brutsaert DL, Sys SU, Gillebert TC. Diastolic failure: pathophysiology and therapeutic implications. J Am Coll Cardiol **22**: 318–325, 1993 (published erratum appears in J Am Coll Cardiol **22**: 1272, 1993).
37) Litwin SE, Grossman W. Diastolic dysfunction as a cause of heart failure. J Am Coll Cardiol **22**: 49A–55A, 1993.
38) Brutsaert DL, Rademakers FE, Sys SU. Triple control of relaxation: implications in cardiac disease. Circulation **69**: 190–196, 1984.

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LETTERS TO THE EDITOR

EFFECTS OF PHYSICAL EXERCISE ON PLASMA CONCENTRATIONS OF SEX HORMONES IN ELDERLY WOMEN WITH DEMENTIA

To the Editor: Physical exercise may slow the functional decline in elderly people and has been associated with a low incidence of dementia.[1] Physical activities have shown favorable effects on cognitive function as well as on neuropsychiatric symptoms and behavioral disturbance in demented subjects,[1,2] the mechanism of which is currently unknown. Because low plasma levels of sex hormones have been implicated in dementia,[3] it is reasonable to hypothesize that physical exercise could elevate plasma sex hormone levels. Here, we report a preliminary study in which daily physical exercise for 3 months increased the plasma levels of sex hormones, including dehydroepiandrosterone (DHEA) and testosterone, in elderly women with dementia. Thirteen women (aged 74–91, mean age ± standard deviation 84 ± 5) living in group homes for the elderly (small-scale facilities providing communal living) located in Nagano Prefecture, Japan, were enrolled. They were diagnosed as having Alzheimer's disease according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, but did not have malnutrition, malignancy, or endocrine disease. Blood sampling and functional assessment were performed at baseline, at the end of a 3-month exercise program, and at the end of a 3-month follow-up period, during which the subjects returned to ordinary sedentary living. The exercise program consisted of stretching and mild resistance training using a chair and a 0.5-kg weight. The exercise was performed as a group, with training for 30 minutes daily under the instruction of a physical therapist twice a week and by other caregiver staff five times a week. Care other than exercise was comparable throughout the study. Fasting blood samples were collected early in the morning before exercise. A commercial laboratory determined plasma levels of estradiol, testosterone, DHEA, DHEA sulfate, and sex hormone–binding globulin, in addition to blood cell counts and blood chemical parameters. Basic activities of daily living (ADLs) were assessed using the Barthel Index and cognitive function using the Mini-Mental State Examination.

At baseline, the subjects showed moderate cognitive impairment and dependency and relatively low sex hormone levels (Table 1). After 3 months of exercise, significant increases were found in plasma levels of testosterone of 18%, estradiol of 38%, and DHEA of 37%, all of which returned to the baseline levels 3 months after cessation of the exercise program. A similar alteration was found in plasma DHEA sulfate level, but the increase by exercise was not statistically significant (mean ± standard error 452 ± 62 ng/mL at baseline, 508 ± 72 ng/mL after exercise, and 464 ± 77 ng/mL after discontinuation. Sex hormone–binding globulin, albumin, and other blood parameters did not change throughout the study (Table 1 and data not shown). Despite the increases in sex hormones after the exercise program, neither Barthel Index nor Mini-Mental State Examination scores changed significantly during the study.

Previous studies[4,5] have shown stimulatory effects of endurance or resistance exercise on circulating hormones in healthy postmenopausal women; metabolic alterations and increased blood flow of endocrine organs via nitric oxide and cyclic adenosine monophosphate production may play a causal role, but hormonal responses in frail or demented women have not been examined. In the present study, plasma levels of estradiol, testosterone, and DHEA were higher after 3 months of physical exercise in elderly women with dementia, whereas cognitive function and basic ADLs did not improve. Given the protective effect of exercise and sex hormones on cognitive impairment, a control sedentary group should be included to examine whether this exercise program might delay cognitive decline. Nevertheless, the finding that exercise can increase plasma sex hormone levels in demented women provides a mechanistic insight into the effect of exercise or physical activities on cognitive impairment. The results of this preliminary study need to be confirmed using larger randomized, controlled trials with longer follow-up periods.

Table 1. Effects of Daily Physical Exercise on Plasma Concentrations of Sex Hormones in Elderly Women with Dementia (N = 13)

Measurement	Baseline	Exercise (3 Months)	Discontinuation (3 Months)
	Mean ± Standard Error of the Mean		
Testosterone, ng/dL	51.4 ± 3.3	60.8 ± 3.3†	47.9 ± 3.9
Estradiol, pg/mL	15.2 ± 1.2	21.0 ± 1.2†	19.4 ± 2.9
Dehydroepiandrosterone, ng/mL	1.84 ± 0.29	2.52 ± 0.41*	1.95 ± 0.27
Sex hormone binding globulin, nmol/L	75.0 ± 6.1	69.1 ± 8.1	68.3 ± 8.3
Barthel Index	75.0 ± 5.4	70.0 ± 7.1	66.5 ± 9.4
Mini-Mental State Examination score	13.9 ± 1.9	13.8 ± 2.0	12.4 ± 2.5

$P < {}^{*}.05$; ${}^{\dagger}.01$ versus baseline using paired t test.

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REFERENCES

1. Colcombe S, Kramer AF. Fitness effects on the cognitive function of older adults: A meta-analytic study. Psychol Sci 2003;14:125–130.
2. Cummings JL. Alzheimer's disease. N Engl J Med 2004;351:56–67.
3. Almeida OP, Barclay L. Sex hormones and their impact on dementia and depression: A clinical perspective. Expert Opin Pharmacother 2001;2:527–535.
4. Copeland JL, Consitt LA, Tremblay MS. Hormonal responses to endurance and resistance exercise in females aged 19 to 69 years. J Gerontol A Biol Sci Med Sci 2002;57A:B158–B165.
5. Kemmler W, Wildt L, Engelke K et al. Acute hormonal responses of a high impact physical exercise session in early postmenopausal women. Eur J Appl Physiol 2003;90:199–209.

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Inhibitory effect of low-dose estrogen on neointimal formation after balloon injury of rat carotid artery

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Abstract

The current regimens of hormone replacement therapy for postmenopausal women, estrogen combined with progestogen, have failed to show beneficial effects for the prevention of atherosclerotic disease. Although the relatively higher dose of estrogen contained in those regimens exerted adverse effects, there are few data examining a lower dose of estrogen in an atherosclerosis model. Therefore, we investigated experimentally whether lower doses of estrogen could inhibit neointimal formation after balloon injury of the rat carotid artery. Ten-week-old Wistar rats were subjected to ovariectomy or sham-operation (n=7). Four days after ovariectomy, rats were implanted with an osmotic mini-pump containing 17-β estradiol (0.2, 1, 2, 10 and 20 μg/kg/day; n=6, 4, 8, 6 and 5, respectively) or placebo (n=10). After 3 days of hormone therapy, balloon injury was performed in the left common carotid artery. Neointimal formation was histologically evaluated 2 weeks after injury. Cross-sectional intimal area and the ratio of intimal area to medial area were dose-dependently reduced by estrogen replacement compared with those in ovariectomized rats without estrogen replacement. The effects of estrogen replacement were identical to those of an angiotensin II type 1 receptor blocker, candesartan. Interestingly, the effect was significant even in rats receiving lower doses of estrogen, in which plasma estradiol concentrations were not increased and the hyperplastic response of the uterus was minimal. These results suggest the efficacy of low-dose estrogen therapy for the protection of atherosclerosis.

Keywords: Estrogen; Low-dose; Neointimal formation

1. Introduction

Previous studies have shown that estrogen administration in ovariectomized animals inhibits the process of atherosclerosis. Different doses of estrogens in combination with or without progestins have decreased the lesion formation in injured vessels or cholesterol-fed animals using rodents, rabbits and swine (Chen et al., 1996; Oparil et al., 1997; Bakir et al., 2000; Chandrasekar and Tanguay, 2000; Finking et al., 2001; Tolbert et al., 2001). Most of the studies, however, have used the estradiol doses of 20 μg/kg/day or higher, which were accompanied by the raised plasma estradiol concentration compared to intact female animals (Chen et al., 1996; Bakir et al., 2000; Tolbert et al., 2001). More importantly, these doses of estrogen (≥20 μg/kg/day of estradiol subcutaneously) elicited adverse effects such as uterine hyperplasia (Bakir et al., 2000; Tolbert et al., 2001; Xu et al., 2003) and dyslipidemia (Joles et al., 1998; Gades et al., 1998; Tomiyoshi et al., 2002). On the other hand, it has been reported that the effect of estradiol on uterine weight was dose-dependent (Kerdelhue and Jolette, 2002) and that low dose estrogen (approximately 3 μg/kg/day of estradiol) could exert its favorable effect on bone metabolism (Chen et al., 2001). Since limited information is

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available on the vascular effect of low dose estrogen therapy, it is intriguing to study whether the lower dose of estrogen could inhibit vascular lesion formation.

In the present study, we hypothesized that lower doses of estrogen could have protective effects on the process of atherosclerosis with minimal adverse effects. To test this hypothesis, we examined neointimal formation of the carotid artery after balloon angioplasty in ovariectomized female rats receiving 10 μg/kg/day or lower doses of estradiol.

2. Materials and methods

2.1. Animals

Ten-week-old female Wistar rats (Oriental Yeast, Tokyo) were used in this study. They were housed in individual cages in a room in which lighting was controlled (12 h on, 12 h off) and room temperature was kept at ≈22 °C. They were given a standard diet and water ad libitum. All the surgical procedures were performed under sterile conditions. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

2.2. Experimental protocols

Rats were randomly divided into 10 groups. Nine groups of rats were subjected to ovariectomy and the other group underwent sham operation (Akishita et al., 1997). After a 4-day recovery period, six groups of ovariectomized rats were subcutaneously implanted with osmotic minipumps (Alzet 2002, 0.5 μl/h; Alza) prefilled with water-soluble 17β-estradiol (0.2, 1, 2, 10 or 20 μg/kg/day; Sigma) or its vehicle (2-hydroxypropyl-β-cyclodextrin; Sigma) under ether anesthesia. To compare the effect of estrogen with that of an angiotensin II type 1 (AT1) receptor blocker, candesartan, the remaining four groups of rats were subcutaneously implanted with an osmotic minipump containing the active metabolite of candesartan, candesartan cilexetil (2, 20 or 200 μg/kg/day; kindly donated by Takeda Chemical Industries, Tokyo) or its vehicle (0.9% saline).

Three days after minipump implantation, balloon injury was performed as previously described (Chen et al., 1996; Nakaoka et al., 1997). General anesthesia was induced by the administration of 90 mg/kg of ketamine intraperitoneally and 15 mg/kg of xylazine intramuscularly. The left carotid artery was exposed and its branches were ligated using 7–0 nylon. After intravenous injection of 75 U/kg of heparin, a portion of the external carotid artery and a portion of the internal carotid artery were cross-clipped using a microclip (2v-clip: S&T, Neuhausen, Switzerland). A 2F Fogarty embolectomy catheter (Baxter, Irvine, CA) was introduced into the artery via the external carotid artery. The common carotid artery was injured by six passes of an embolectomy catheter inflated with 0.2 ml of air. The portion proximal to the incision was ligated with 7–0 nylon, the cross-clip was released and the common carotid artery was reperfused.

2.3. Measurement of hormones and lipids

Blood sampling was performed at sacrifice, after a 16-h overnight fast, to measure serum concentrations of estradiol and progesterone, serum lipids and other biochemical parameters. Serum estradiol, estrone and progesterone concentrations were measured by sensitive radioimmunoassay (Hashimoto et al., 2002). Serum total cholesterol and triglyceride concentrations were measured enzymatically, and serum high-density lipoprotein cholesterol concentration was measured by heparin-Ca^{2+} Ni^{2+} precipitation method (Hashimoto et al., 2002).

2.4. Morphometrical analysis of the balloon-injured carotid artery

A portion of the left common carotid artery was harvested at 14 days after balloon injury. The artery was perfusion- and pressure-fixed at 100 mm Hg using 10% neutral formalin buffer and then paraffin-embedded. Five round cross-sections per 1.5-cm length of artery specimens were stained with *Elastica van Gieson staining*, and photographed. Cross-sectional areas of the intima and the media were measured using an image analyzing software package (Scion Image, shared NIH software). The average of five sections was used for analysis as the value of each animal.

2.5. Data analysis

Values are expressed as mean±S.E.M. in the text, table and figures. Data were analyzed by one-factor analysis of variance (ANOVA) followed by Newman–Keuls' multiple comparison test. Differences with a value of $P<0.05$ were considered statistically significant.

3. Results

Sixty-five rats were set up and allocated to each group. Four rats were excluded because of failure of intervention. Estrogen replacement in ovariectomized rats increased serum concentration of estradiol dose-dependently, and replacement of 2 μg/kg/day estradiol achieved a concentration comparable to that in sham-operated rats (Table 1). In all groups, the serum concentration of estrone was below the detection limit (data not shown) and that of progesterone was unchanged. With respect to the lipid profile, the concentration of total cholesterol, triglyceride and high-density lipoprotain (HDL) cholesterol were increased in rats

Table 1
Blood pressure, serum lipids, plasma hormone concentrations and body and uterus weight after balloon injury of left carotid arteries of female Wistar rats

	Sham	Ovariectomy+17β-estradiol (μg/kg/day)						Ovariectomy+TCV-116 (μg/kg/day)		
		0	0.2	1	2	10	20	0	2	20
No. of rats	7	10	6	4	8	6	5	4	4	4
SBP (mm Hg)	121±4	113±7	123±2	120±5	127±2	121±4	121±4	121±7	122±7	116±8
T.chol (mg/dl)	76±9	75±5	86±4	78±10	84±6	96±5[a]	113±3[b]	79±2	89±4	81±8
HDL-C (mg/dl)	20±2	21±3	20±2	16±3	23±2	27±1	30±1[a]	17±2	21±2	22±2
Triglyceride (mg/dl)	41±6	53±8	46±9	64±16	91±13[a]	87±10[a]	153±31[b]	64±11	25±6	35±10
Estradiol (pg/ml)	19±4[b]	8±1	9±1	12±2	20±2[b]	54±5[b]	96±3[b]	11±3	11±1	14±2
Progesterone (ng/ml)	20±5	13±2	6±3	21±5	9±2	11±3	5±2	16±4	21±6	15±6
Body weight (g)	269±6	282±8	281±8	260±6	264±6	257±5[a]	263±7	285±10	290±5	290±3
Uterus (mg)	661±102[b]	174±29	321±23	577±46[b]	511±76[b]	–	–	148±22	149±5	156±7

Values are expressed as mean±S.E.M. SBP, systolic blood pressure; T.chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; –, not examined.
[a] $P<0.05$ vs. OVX+0 μg/kg/day of 17β-estradiol.
[b] $P<0.01$ vs. OVX+0 μg/kg/day of 17β-estradiol.

receiving higher doses of estrogen, as previously reported (Gades et al., 1998; Joles et al., 1998; Tomiyoshi et al., 2002), whereas those were unchanged in rats receiving 2 μg/kg/day or a lower dose of estrogen. The body weight of rats treated with higher doses was significantly lower than that in rats without estrogen replacement. In contrast, uterine weight in rats receiving lower doses of estrogen was greater than that in rats without estrogen.

Morphometric analysis showed that the neointimal area of the carotid artery was dose-dependently decreased by estrogen replacement (Figs. 1 and 2). As shown in Fig. 2, neointimal formation was sufficiently attenuated even in rats treated with 0.2 μg/kg/day of estradiol compared to that in ovariectomized rats without estrogen replacement. The inhibitory effect of estrogen on neointimal formation was compared with that of candesartan because the effects of AT1 receptor blockers including candesartan have been established (Kim et al., 2002; Liu et al., 2002; Nozawa et al., 1999; Tazawa et al., 1999). The effect of 20 μg/kg/day estradiol was more potent than that of subdepressor dose of candesartan (20 μg/kg/day) and was as potent as that of 200 μg/kg/day candesartan; a dose that lowered blood pressure and body weight as well as neointimal formation (intima/media ratio was 0.66±0.07, data not shown). Importantly, the effect of 2 μg/kg/day or a lower dose of estradiol on neointima formation was comparable to that of 20 μg/kg/day candesartan (Fig. 2). Medial area was not different among all groups of rats. Small non-significant differences in several measurements between the control for estrogen and that for candesartan were likely to be due

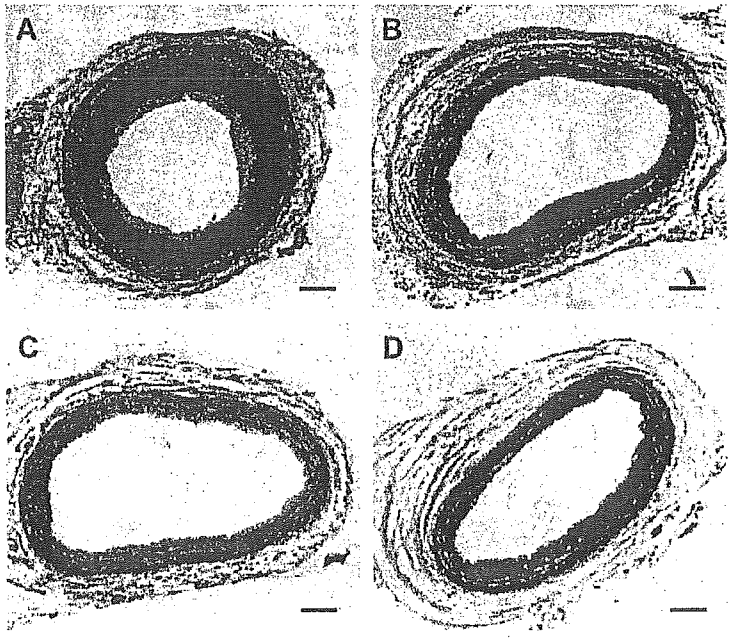

Fig. 1. Representative cross-sections of the rat carotid artery 2 weeks after balloon injury (elastica van gieson staining, magnification ×100). Rats were treated with 20% cyclodextrin vehicle (A), 0.2 μg/kg/day of 17-β estradiol (B), 20 μg/kg/day of 17-β estradiol (C) and 20 μg/kg/day of candesartan (D). Bars: 100 μm.

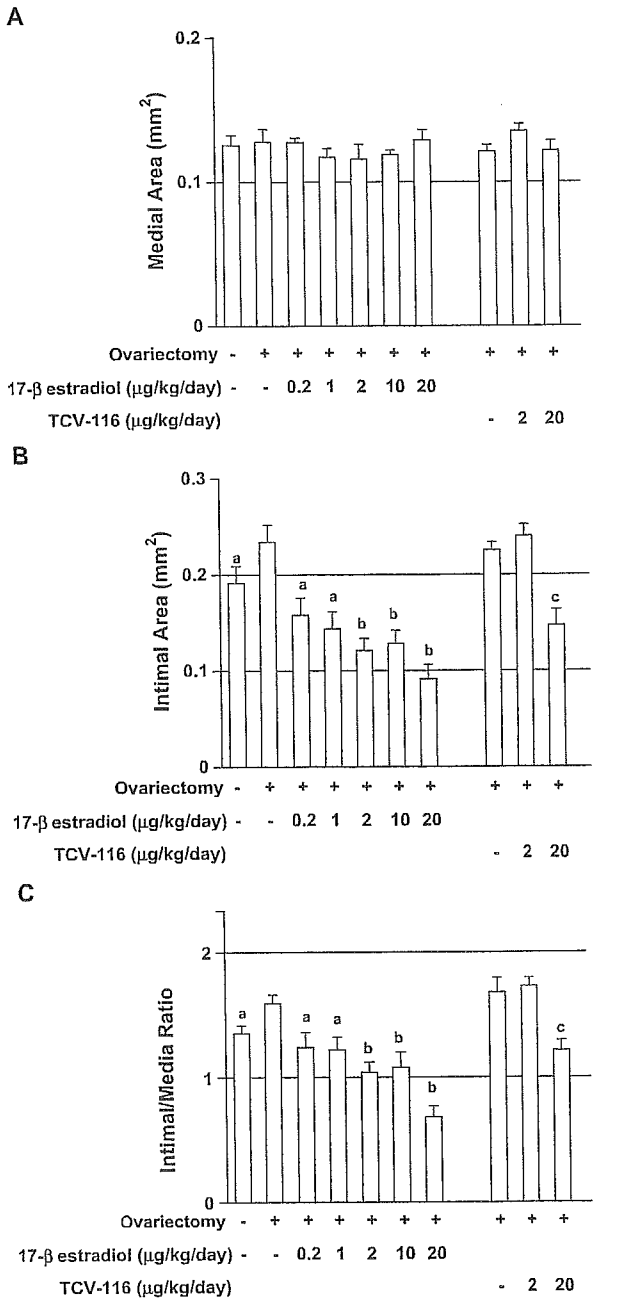

Fig. 2. Morphometric analyses of intimal area (A), medial area (B) and intima/media area ratio (C) in the carotid artery 2 weeks after balloon injury. The results are expressed as mean±S.E.M. $^{a}P<0.05$, $^{b}P<0.01$ vs. ovariectomized rats without 17-β estradiol, $^{c}P<0.01$ vs. ovariectomized rats without candesartan.

to the variation of the measurements rather than the effect of vehicle for each group.

4. Discussion

This study showed that subcutaneous administration of 2 μg/kg/day or lower doses of estradiol inhibited neointimal formation after vascular injury with minimal adverse effects on the uterus and lipid metabolism, suggesting the efficacy of lower doses of hormone replacement therapy for the prevention of atherosclerosis.

Estrogen has been reported to inhibit neointimal formation after vascular injury in rodents using balloon angioplasty of the rat carotid artery (Bakir et al., 2000; Chen et al., 1996; Oparil et al., 1997, 1999), cuff placement around the rat femoral artery (Akishita et al., 1997) and ligation of the mouse carotid artery (Tolbert et al., 2001). Oparil and her colleagues have shown using the rat carotid balloon-injury model that subcutaneous administration of 20 μg/kg/day estradiol reduced neointimal formation by more than 50% compared to that without estradiol treatment (Chen et al., 1996; Oparil et al., 1997, 1999; Bakir et al., 2000). In their studies, plasma estradiol levels in estrogen-replaced rats (135.0±5.7 pg/ml, Chen et al., 1996, or 32.0±4.8 pg/ml, Bakir et al., 2000) were higher than those in intact female rats (51.9±5.8 pg/ml, Chen et al., 1996, or 25±6.9 pg/ml, Bakir et al., 2000). In the present study, administration of 10 or 20 μg/kg/day estradiol in ovariectomized rats inhibited neointimal formation with the increased plasma estradiol concentration beyond that in sham-operated rats as well. These results suggest that the estradiol doses used in the previous studies (>10 μg/kg/day) may be relatively high although plasma estradiol concentration fluctuates in rats with the estrous cycle (ranged from 16±2 to 39±7 pg/ml, Anisimov and Okulov, 1980, or from 1±1 to 44±15 pg/ml, Hawkins et al., 1975), and changes with development and age (Meijs-Roelofs et al., 1975). In contrast, replacement of 2 μg/kg/day estradiol achieved serum estradiol concentrations comparable to those in sham-operated rats in the present study. Replacement of 1 μg/kg/day or a lower dose of estradiol did not increase the serum estradiol concentration. However, the inhibition of neointimal formation was significant at the lower doses and was comparable to the effect of 20 μg/kg/day of candesartan (Fig. 2). Moreover, 1 μg/kg/day or a lower dose of estradiol did not increase the serum triglyceride concentration, and 0.2 μg/kg/day of estradiol caused the minimal and non-significant increase of uterus weight. This could be a new finding with respect to the adverse effects on lipid profiles and uterus. Taken these findings together, a local effect of estrogen replacement on organs or cells was observed even if circulating estrogen was not elevated, providing some hints on determining the dose of hormone replacement therapy.

In the present study, we did not demonstrate the mechanisms by which estrogen inhibited neointimal formation. Previous reports have shown that re-endothelialization (White et al., 1997), preservation of endothelial survival (Sudoh et al., 2001) and function (White et al., 1997), inhibition of smooth muscle cell proliferation (Akishita et al., 1997) and inhibition of fibroblast proliferation and differentiation in the adventitia (Oparil et al., 1999) contribute to the effect of estrogen on the response to

vascular injury. Stimulation of nitric oxide synthesis as well as modulation of other vasoactive substances has been implicated in these effects, although activation of endothelial nitric oxide synthase may play a major role (Chambliss and Shaul, 2002). Further investigation is needed to elucidate the contribution and interaction of these factors in the effects of lower doses of estrogen on neointimal formation.

Recent randomized trials (Hulley et al., 1998; Rossouw et al., 2002) have suggested that hormone replacement therapy with the standard regimen should not be recommended for postmenopausal women. Improvement of the regimen, such as the dose, route (oral or subcutaneous) or schedule (continuous or cyclic), could resolve the adverse effects of hormone replacement therapy, although few data are currently available (Grodstein et al., 2000; Jick et al., 1996; Hashimoto et al., 2002; Wakatsuki et al., 2003, 2004). Direct comparisons of animal studies to clinical studies are inadequate because several major differences can be pointed including route of administration, duration of the treatment, cardiovascular risk profile of subjects and body fat distribution. However, our experimental result that lower doses of estrogen inhibited the response to vascular injury with relatively small adverse effects may imply the potential efficacy of low dose hormone replacement therapy in postmenopausal women.

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References

Akishita, M., Ouchi, Y., Miyoshi, H., Kozaki, K., Inoue, S., Ishikawa, M., Eto, M., Toba, K., Orimo, H., 1997. Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: effects on migration and proliferation of vascular smooth muscle cells. Atherosclerosis 130, 1–10.

Anisimov, V.N., Okulov, V.B., 1980. Effect of ageing on concentration of estradiol in serum and the epidermal G2 chalone in vaginal mucosa of rats. Exp. Gerontol. 15, 87–91.

Bakir, S., Mori, T., Durand, J., Chen, Y.F., Thompson, J.A., Oparil, S., 2000. Estrogen-induced vasoprotection is estrogen receptor dependent: evidence from the balloon-injured rat carotid artery model. Circulation 101, 2342–2344.

Chambliss, K.L., Shaul, P.W., 2002. Estrogen modulation of endothelial nitric oxide synthase. Endocr. Rev. 23, 655–686.

Chandrasekar, B., Tanguay, J.F., 2000. Local delivery of 17-beta-estradiol decreases neointimal hyperplasia after coronary angioplasty in a porcine model. J. Am. Coll. Cardiol. 36, 1972–1978.

Chen, S.J., Li, H., Durand, J., Oparil, S., Chen, Y.F., 1996. Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. Circulation 93, 577–584.

Chen, J.L., Yao, W., Frost, H.M., Li, C.Y., Setterberg, R.B., Jee, W.S.S., 2001. Bipedal stance exercise enhances antiresorption effects of estrogen and counteracts its inhibitory effect on bone formation in sham and ovariectomized rats. Bone 29 (2), 126–133.

Finking, D., Krauss, N., Romer, S., Eckert, S., Lenz, C., Kamenz, J., Menke, A., Brehme, U., Hanke, H., 2001. 17beta-estradiol, gender independently, reduces atheroma development but not neointimal proliferation after balloon injury in the rabbit aorta. Atherosclerosis 154, 39–49.

Gades, M.D., Stern, J.S., van Goor, H., Nguyen, D., Johnson, P.R., Kaysen, G.A., 1998. Estrogen accelerates the development of renal disease in female obese Zucker rats. Kidney Int. 53, 130–135.

Grodstein, F., Manson, J.E., Colditz, G.A., Willett, W.C., Speizer, F.E., Stampfer, M.J., 2000. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. Ann. Intern. Med. 133, 933–941.

Hashimoto, M., Miyao, M., Akishita, M., Hosoi, T., Toba, K., Kozaki, K., Yoshizumi, M., Ouchi, Y., 2002. Effects of long-term and reduced-dose hormone replacement therapy on endothelial function and intima-media thickness in postmenopausal women. Menopause 9, 58–64.

Hawkins, R.A., Freedman, B., Marshall, A., Killen, E., 1975. Oestradiol-17 beta and prolactin levels in rat peripheral plasma. Br. J. Cancer 32, 179–185.

Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., Vittinghoff, E., 1998. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/Progestin Replacement Study (HERS) Research Group. JAMA 280, 605–613.

Jick, H., Derby, L.E., Myers, M.W., Vasilakis, C., Newton, K.M., 1996. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogens. Lancet 348, 981–983.

Joles, J.A., van Goor, H., Koomans, H.A., 1998. Estrogen induces glomerulosclerosis in analbuminemic rats. Kidney Int. 53, 862–868.

Kerdelhue, B., Jolette, J., 2002. The influence of the route of administration of 17beta-estradiol, intravenous (pulsed) versus oral, upon DMBA-induced mammary tumour development in ovariectomised rats. Breast Cancer Res. Treat. 73, 13–22.

Kim, S., Izumi, Y., Izumiya, Y., Zhan, Y., Taniguchi, M., Iwao, H., 2002. Beneficial effects of combined blockade of ACE and AT1 receptor on intimal hyperplasia in balloon-injured rat artery. Arterioscler. Thromb. Vasc. Biol. 22, 1299–1304.

Liu, H.W., Iwai, M., Takeda-Matsubara, Y., Wu, L., Li, J.M., Okumura, M., Cui, T.X., Horiuchi, M., 2002. Effect of estrogen and AT1 receptor blocker on neointima formation. Hypertension 40, 451–457. (discussion 448-450).

Meijs-Roelofs, H.M., Uilenbroek, J.T., De Greef, W.J., De Jong, F.H., Kramer, P., 1975. Gonadotrophin and steroid levels around the time of first ovulation in the rat. J. Endocrinol. 67, 275–282.

Nakaoka, T., Gonda, K., Ogita, T., Otawara-Hamamoto, Y., Okabe, F., Kira, Y., Harii, K., Miyazono, K., Takuwa, Y., Fujita, T., 1997. Inhibition of rat vascular smooth muscle proliferation in vitro and in vivo by bone morphogenetic protein-2. J. Clin. Invest. 100, 2824–2832.

Nozawa, Y., Matsuura, N., Miyake, H., Yamada, S., Kimura, R., 1999. Effects of TH-142177 on angiotensin II-induced proliferation, migration and intracellular signaling in vascular smooth muscle cells and on neointimal thickening after balloon injury. Life Sci. 64, 2061–2070.

Oparil, S., Levine, R.L., Chen, S.J., Durand, J., Chen, Y.F., 1997. Sexually dimorphic response of the balloon-injured rat carotid artery to hormone treatment. Circulation 95, 1301–1307.

Oparil, S., Chen, S.J., Chen, Y.F., Durand, J.N., Allen, L., Thompson, J.A., 1999. Estrogen attenuates the adventitial contribution to neointima formation in injured rat carotid arteries. Cardiovasc. Res. 44, 608–614.

Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C., Stefanick, M.L., Jackson, R.D., Beresford, S.A., Howard, B.V., Johnson, K.C., Kotchen, J.M., Ockene, J., 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative Randomized Controlled Trial. JAMA 288, 321–333.

Sudoh, N., Toba, K., Akishita, M., Ako, J., Hashimoto, M., Iijima, K., Kim, S., Liang, Y.Q., Ohike, Y., Watanabe, T., Yamazaki, I., Yoshizumi, M., Eto, M., Ouchi, Y., 2001. Estrogen prevents oxidative stress-induced endothelial cell apoptosis in rats. Circulation 6;103 (5), 724–729.

Tazawa, S., Nakane, T., Chiba, S., 1999. Angiotensin II type 1 receptor blockade prevents up-regulation of angiotensin II type 1A receptors in rat injured artery. J. Pharmacol. Exp. Ther. 288, 898–904.

Tolbert, T., Thompson, A., Bouchard, P., Oparil, S., 2001. Estrogen-induced vasoprotection is independent of inducible nitric oxide synthase expression. Evidence from the mouse carotid artery ligation model. Circulation 104, 2740–2745.

Tomiyoshi, Y., Sakemi, T., Aoki, S., Miyazono, M., 2002. Different effects of castration and estrogen administration on glomerular injury in spontaneously hyperglycemic Otsuka Long–Evans Tokushima Fatty (OLETF) rats. Nephron 92, 860–867.

Wakatsuki, A., Okatani, Y., Ikenoue, N., Shinohara, K., Watanabe, K., Fukaya, T., 2003. Effect of lower dose of oral conjugated equine estrogen on size and oxidative susceptibility of low-density lipoprotein particles in postmenopausal women. Circulation 108, 808–813.

Wakatsuki, A., Ikenoue, N., Shinohara, K., Watanabe, K., Fukaya, T., 2004. Effect of lower dosage of oral conjugated equine estrogen on inflammatory markers and endothelial function in healthy postmenopausal women. Arterioscler. Thromb. Vasc. Biol. 24 (3), 571–576.

White, C.R., Shelton, J., Chen, S.J., Darley-Usmar, V., Allen, L., Nabors, C., Sanders, P.W., Chen, Y.F., Oparil, S., 1997. Estrogen restores endothelial cell function in an experimental model of vascular injury. Circulation 96, 1624–1630.

Xu, Y., Arenas, I.A., Armstrong, S.J., Davidge, S.T., 2003. Estrogen modulation of left ventricular remodeling in the aged heart. Cardiovasc. Res. 57, 388–394.

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ORIGINAL ARTICLE

Improvement of inappropriate prescribing and adverse drug withdrawal events after admission to long-term care facilities

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Background: The objectives of this study were to determine whether medications, particularly inappropriate prescribing, would be reduced after admission to long-term care facilities, and whether adverse drug withdrawal events (ADWEs) would occur in relation to discontinuation of medications.

Methods: The study consists of a retrospective survey using medical chart review in five health service facilities for the elderly in Japan. All the patients who were admitted to the facilities between January 2001 and December 2002 ($N = 627$) were participants in the study. Medications taken on admission, at 1 month and 3 months after admission, and events (significant worsening of the disease status, accidents, new symptoms and signs, and other acute events) during a 3-month period were recorded. Inappropriate prescribing was determined using Beers' criteria with some modification. ADWEs were determined using the Naranjo causality algorithm.

Results: On admission, the patients were taking 3.5 ± 2.5 (mean $\pm$ SD) drugs. One month later, the number of prescribed drugs was decreased by 17% ($P < 0.01$ vs on admission), but did not show an additional reduction 3 months later. Inappropriate prescribing was found in 10% of the patients taking drugs on admission, but the number of inappropriately prescribed medications was reduced by 33% after 1 month. Of 105 events recorded, only five (2% of the patients with drug reduction) were considered ADWEs; three cases of confusion, a case of depression, and a case of hyperglycemia, following discontinuation of psychotropic drugs, antidepressants and a sulfonylurea, respectively.

Conclusion: Adverse drug withdrawal events were not frequent despite the significant reduction of medications after admission to long-term care facilities. This might be because the rate of reduction was relatively high for inappropriately prescribed medications.

Keywords: adverse drug reaction, long-term care, medical expense, medical injury, pharmacotherapy.

Introduction

Adverse drug reactions in elderly people increase with age,[1–3] with most being attributable to medication errors that are preventable.[3,4] Age-dependent changes in pharmacokinetics and pharmacodynamics, polypharmacy and non-compliance related to patients' functional

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decline may play a role.[1,3] In particular, polypharmacy resulting from multiple pathology in elderly people is a critical problem leading to adverse drug reactions.[1–3] To prevent polypharmacy, review of prescriptions is essential according to evidence-based medicine and criteria for inappropriate prescribing.[5,6] In fact, inappropriate use of medication in elderly people has been reported to be as frequent as 16% to 25%.[7–9]

Conversely, discontinuation of medications to improve polypharmacy or inappropriate prescribing may induce adverse drug withdrawal events (ADWEs),[10] although the net effect on adverse drug reactions can be favorable in elderly outpatients.[11] Fixed payment insurance systems restrict medication use, possibly leading to a reduction of inappropriate prescribing and/or an increase of ADWEs. In health service facilities for the elderly in Japan, where functional training and nursing/personal care are provided under long-term care insurance,[12] a fixed payment system including prescribing of medication is applied. Accordingly, it is hypothesized that prescribed drugs, particularly inappropriate prescribing, would be reduced after admission to the facilities, and that ADWEs would occur in relation to discontinuation of medications. To test this hypothesis, we performed a retrospective chart review of a total of 627 patients in five health service facilities for the elderly, and found that prescribed drugs can be reduced with few ADWEs in such a frail elderly population with chronic diseases.

Methods

Sample and data collection

The data were derived from five health service facilities for the elderly (Mahoroba-no-Sato, Nagano; Moeu-no-Sato, Nagano; Himawari-En, Fukuoka; Millenium-Sakuradai, Tokyo; Mizukusaki-En, Tokyo) in Japan. Institutional medical charts were reviewed for all the patients admitted between January 2001 and December 2002. Diagnoses of each patient were not recorded because they were unclear from the institutional charts, but Alzheimer's disease, cerebrovascular disease and osteoporosis were the main causes of disability in each institution. The average basic activities of daily living, as measured by the Barthel index, were 70–80 points out of 100 points according to the institutions. Medications that the patients were taking on admission and prescribed drugs 1 month and 3 months after admission were recorded. Similarly, all the events (significant worsening of the disease status, accidents, new symptoms and signs, and other acute events) during a 3-month period were recorded. The institutions that managed the patients before admission were categorized as acute care hospitals, outpatient clinics (home), sanitarium-type wards, special nursing homes for the elderly and health service facilities for the elderly. Patients with voluntary discharge within 3 months excluding cases of death or transfer to another hospital were excluded, and a total of 627 patients were analyzed. The director of each institution gave written approval to the participation in this study. The study protocol was approved by the committee on ethics and the institutional review board of Kyorin University School of Medicine.

Analysis

Inappropriately prescribed medications were determined using an updated version of the list developed by Beers with some modification.[5] Basically, we followed the list by Sloane *et al.* in which several drugs were excluded from Beers' list in consultation with Dr Beers,[5,9] reflecting changes in pharmacotherapy, but we included digoxin at more than 0.125 mg/day and oral iron at more than 325 mg/day in the list because these dosages were recorded in the medical chart. In this study, diagnosis-related inappropriate prescribing was excluded,[3] as in the study by Sloane *et al.* because the institutional chart did not include all the diagnoses of the patients.[9]

All the events were reviewed by a consultant geriatrician, and ADWEs were determined using the Naranjo causality algorithm.[13] Because detailed information, such as the effect of re-administration was lacking in most cases, a probability scale ≥ 1 (possible, probable or definite) was considered to indicate an ADWE.

The data in the text and the tables are expressed as means ± SD unless otherwise specified. Changes in the number of prescribed drugs after admission were analyzed using paired *t*-test. Differences between the groups were analyzed using ANOVA followed by Newman-Keuls' test.

Results

Number of prescribed drugs

The patients were taking 3.5 ± 2.5 drugs when admitted to the facilities (Table 1). Forty-six patients (7.3%) were not taking any drug, while 50 patients (8.0%) were on eight or more drugs. Women were taking fewer drugs than men. This sex difference seemed independent of age, although a statistically significant difference was found only at 80–89 years of age when the patients were categorized by age groups (Table 1). Interestingly, patients of 80 years or older were taking fewer drugs than those younger than 70 years, in contrast to a previous finding that the number of prescribed drugs increased according to age.[2,14,15]

As shown in Table 2, the mean number of prescribed drugs had decreased by 0.6 (17%) 1 month

Table 1 Number of drugs taken on admission according to sex and age

	All	Men	Women	*P* for sex difference
Total	3.5 ± 2.5 (627)	4.2 ± 2.8 (177)	3.3 ± 2.4 (450)	< 0.01
≤ 69 years	4.4 ± 3.1 (36)	4.6 ± 3.5 (19)	4.2 ± 2.6 (17)	0.70
70–79 years	4.0 ± 2.6 (131)	4.6 ± 3.0 (43)	3.7 ± 2.3 (88)	0.08
80–89 years	3.3 ± 2.3* (316)	4.0 ± 2.6 (81)	3.0 ± 2.2 (235)	0.02
≥ 90 years	3.5 ± 2.7* (144)	4.2 ± 2.4 (34)	3.2 ± 2.8 (110)	0.08

*$P < 0.05$ versus ≤ 69 years by Newman-Keuls' test.
Data are expressed as mean ± SD. Number of subjects is indicated in parentheses.

Table 2 Changes in number of prescribed drugs after admission to health service facilities for the elderly

	No. of subjects	On admission	After 1 month	After 3 months
Total	627	3.5 ± 2.5	2.9 ± 2.2*	3.0 ± 2.1*
Type of institution before admission				
Acute care hospital	115	4.8 ± 3.3†	4.2 ± 2.9*†	4.1 ± 2.7†
Outpatient	200	3.6 ± 2.3	2.8 ± 1.8*	2.9 ± 2.0
Special nursing home	24	3.3 ± 2.1	2.5 ± 1.7*	2.6 ± 1.8*
Sanitarium-type ward	188	3.1 ± 2.3	2.6 ± 1.9*	2.6 ± 1.9*
Health service facility	100	2.6 ± 1.8	2.4 ± 1.6*	2.5 ± 1.7*
Facility				
A	83	4.9 ± 3.4	4.6 ± 3.0	4.6 ± 2.4
B	80	4.2 ± 2.8	3.9 ± 2.4*	4.0 ± 2.5
C	39	4.1 ± 2.7	2.4 ± 1.7*	2.2 ± 1.4*
D	172	3.2 ± 1.9	2.4 ± 1.5*	2.4 ± 1.5*
E	253	3.0 ± 2.2	2.6 ± 1.9*	2.5 ± 1.9
Event				
No	517	3.5 ± 2.5	2.8 ± 2.0*	2.8 ± 2.1*
Yes	104	3.6 ± 2.7	3.4 ± 2.5***	3.7 ± 2.2**‡

*$P < 0.01$ versus on admission by paired *t*-test; **$P < 0.01$ versus after 1 month by paired *t*-test; †$P < 0.01$ versus other types of institution by Newman-Keuls' test; ***$P < 0.05$; ‡0.01 versus Event (–) by Newman-Keuls' test.
Data are expressed as mean ± SD.

after admission ($P < 0.01$ versus on admission), but did not show an additional reduction 3 months after admission. A significant reduction was seen at 1 month irrespective of the type of institution that had managed the patients before admission, although the number of drugs on admission and the degree of reduction differed between the types of institutions. However, there was a large variation in the reduction of prescribed drugs between the facilities, presumably due to differences in the overall philosophy of the attending physicians and the disease and/or functional status of the patients. Patients with and without events during a 3-month period were analyzed separately (Table 2). They were taking a comparable number of medications on admission. The number of drugs in the patients with events was not significantly decreased at 1 month, and was rather increased at 3 months after admission because in many cases additional drugs were prescribed for treatment of events.

Discontinued drugs and inappropriate prescribing

Categorized by therapeutic class, discontinuation was frequent with neuropsychologic (121 cases), gastrointestinal (116 cases) and cardiovascular (94 cases) drugs, followed by metabolic/endocrine drugs (36 cases). Anti-ulcer drugs (44 cases) including H2 blockers and prostaglandin analogs, antipsychotics (35 cases), antihypertensives (33 cases) including calcium channel blockers, β blockers and angiotensin converting enzyme inhibitors, hypnotics (31 cases), laxatives (31 cases) and non-steroidal anti-inflammatory drugs (22 cases) were frequently withdrawn.

On admission, inappropriate prescribing was seen in 58 patients (10.0% of 581 patients taking drugs). Ticlopidine, digoxin at more than 0.125 mg/day and oxybutynin were prescribed in five or more cases (Table 3). Inappropriately prescribed medications were reduced by 33% 1 month after admission, and did not change

Table 3 Number of inappropriately prescribed drugs on admission and 1 month after admission

Medication	On admission	After 1 month
Ticlopidine	36	25
Digoxin†	11	8
Oxybutynin	5	4
Amitriptyline	4	2
Benzodiazepines‡	3	1
Disopyramide	1	1
Indomethacin	1	1
Total	61	41

†More than 0.125 mg/day; ‡Flurazepam, Chlordiazepoxide and Diazepam

thereafter (data not shown). The reduction was not restricted to specific drugs.

Events during admission

A total of 104 events were seen in 16.7% of the patients during a 3-month admission period. Frequent events (nine cases or more) were new occurrences or worsening of psychological disorders (14 cases); gastrointestinal symptoms (12 cases); respiratory problems, including aspiration, pneumonia and respiratory failure (10 cases); pyrexia and infection other than pneumonia (10 cases); and falls and fractures (nine cases).

Five cases of ADWEs were found in 2.2% of 230 patients with drug reduction. These included three cases of confusion following discontinuation of psychotropic drugs, a case of depression following discontinuation of antidepressants and a case of hyperglycemia following discontinuation of a sulfonylurea.

Subgroups analyses were performed to examine the bias effect on events. The rates of events by type of institutions before admission were 24.5% in the subjects from acute care hospitals, 18.1% in those from outpatient clinics (not significant compared to other groups) and 13.1% in those from other types of institutions ($P < 0.05$ versus the subjects from acute care hospitals). Specific types of events were not related to the higher rate of events in the subjects from acute care hospitals, suggesting that unstable conditions of these patients may play a role. Of five cases with ADWEs, three were found in the subjects from outpatient clinics, one from special nursing homes and one from sanitarium-type wards. Thus, it is likely that possible non-compliance in outpatients or types of institutions before admission did not influence the principal results concerning ADWEs.

The subjects in facilities A and B (Table 2), in which significant drug reduction was not observed, showed a higher rate of events than those in other facilities (28.1% versus 12.9%, $p < 0.001$). This result indicates that adverse drug reactions associated with polypharmacy would have been included in these events, or additional drugs would have been prescribed for treatment of events, although no specific type of events was noted regarding the difference between the facilities. No ADWEs were found in the subjects in facilities A and B, presumably relating to the continuation of medications.

Discussion

The present study showed that the number of prescribed drugs was significantly decreased within 1 month after admission to health service facilities for the elderly. Discontinuation was not limited to inappropriate prescribing, but a larger proportion of inappropriately prescribed medications were discontinued compared to the total reduction of prescribed drugs (33% versus 17%). ADWEs were not frequent, being found in only 2.2% of the patients with drug reduction, while unrelated events occurred in 16.7% of the total patients.

Reflecting on the high incidence of polypharmacy and adverse drug reactions in elderly patients,[1–3,14] the principal finding of the present study that prescribed drugs can be reduced safely in frail elderly patients provides important information on pharmacotherapy. Every physician may make an effort to prescribe the minimum number of drugs, but a patient's long history of illness results in the accumulation of prescribed drugs together with the uncertain efficacy of the drugs. Consequently, the necessity of each medication should be reviewed regularly according to evidence-based medicine and criteria for inappropriate prescribing.[5,6] There is a great opportunity to reconsider prescriptions when attending physicians and/or the insurance system change, as was the case with the present study.

The number of prescribed drugs on admission in this study was smaller than that found in the geriatric ward of our university hospital and that found in residential care/assisted living facilities in the USA.[2,9] This may be because nearly half of the subjects were admitted from long-term care hospitals or facilities, and thus, prescribed drugs had already been restricted. In fact, patients from acute care hospitals were taking more drugs than those from other types of institution. It is interesting that an older age was associated with a smaller number of prescribed drugs, and this did not change when the data were analyzed according to the type of institution from which the patients had come (data not shown). This finding is inconsistent with previous observations in hospitalized or community-dwelling patients,[2,14,15] but is reasonable to prevent non-compliance and adverse drug reactions. At the same time, however, the age-related decrease in medications may involve possible discrimination towards very old people. The smaller number of prescribed drugs in women and discontinuation of medications after

admission in this study are inconsistent with previous reports,[16,17] and may imply age and sex discrimination, although discontinuation seemed successful in this study. Thus, the discrimination issue should also be taken into consideration concerning pharmacotherapy in older people.

In the present study, ADWEs were fewer than the previously reported study in which 26% of cases of discontinuation led to ADWEs in elderly outpatients during a 4-month period.[10] One of the reasons that ADWEs were rare in the present study might be that the rate of reduction was relatively high for inappropriately prescribed medications, although most of the attending physicians did not know the criteria for inappropriate prescribing such as Beers' list.[5] Another reason is that consultant physicians or geriatricians made decisions on prescriptions, based on the disease and functional status of each patient. In fact, most of discontinued drugs were not on the list of inappropriate prescribing, implying that unnecessary drugs had been prescribed before admission to long-term care facilities. In addition, it is possible that we missed ADWEs that progressed very slowly and manifested after the follow-up period of 3 months. We also failed to address the effect of prophylactic medications such as antiplatelet and lipid-lowering agents.

It should be kept in mind that the present results were obtained in a frail elderly population admitted to long-term care facilities where most of the subjects were in a stable state with chronic illness.[12] However, as a model to investigate the effect of drug reduction in elderly people, the present findings will add new insight into pharmacotherapy in the elderly, and should be confirmed in different settings such as hospitals and outpatient clinics. Obviously, medications for acute illness should neither be decreased, nor should physicians hesitate to initiate them even in very old patients, and in fact, prescribed drugs were increased in the patients with events during admission in this study. To safely apply the findings of the present study to clinical practice, knowledge of the criteria for inappropriate prescribing should be widely distributed, and blanket discontinuation of drugs must be avoided. In the present study, we used the Beers' criteria to determine inappropriately prescribed medications because corresponding criteria do not exist in Japan.[5] Consequently, we failed to check many inappropriate drugs that are used in Japan but are not on the Beers' list or sold in the USA. Future investigation using the Japanese criteria for inappropriate prescribing, which the Japan Geriatrics Society is going to establish, will add more information. In Japan, the fixed payment insurance system has begun to cover elderly patients, with the expansion of the elderly population and medical expenses. Therefore, it is essential to establish an effective and safe way to refine the use of medication in elderly people in terms of prevention of adverse drug reactions and ageism.

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References

1 Hanlon JT, Shimp LA, Semla TP. Recent advances in geriatrics: drug-related problems in the elderly. *Ann Pharmacother* 2000; **34**: 360–365.
2 Akishita M, Toba K, Nagano K, Ouchi Y. Adverse drug reactions in older people with dementia. *J Am Geriatr Soc* 2002; **50**: 400–401.
3 Rothschild JM, Bates DW, Leape LL. Preventable medical injuries in older patients. *Arch Intern Med* 2000; **160**: 2717–2728.
4 Phillips DP, Christenfeld N, Glynn LM. Increase in US medication-error deaths between 1983 and 1993. *Lancet* 1998; **351**: 643–644.
5 Beers MH. Explicit criteria for determining potentially inappropriate medication use by the elderly. An update. *Arch Intern Med* 1997; **157**: 1531–1536.
6 Hanlon JT, Schmader KE, Samsa GP *et al.* A method for assessing drug therapy appropriateness. *J Clin Epidemiol* 1992; **45**: 1045–1051.
7 Spore DL, Mor V, Larrat P, Hawes C, Hiris J. Inappropriate drug prescriptions for elderly residents of board and care facilities. *Am J Public Health* 1997; **87**: 404–409.
8 Hanlon JT, Schmader KE, Boult C *et al.* Use of inappropriate prescription drugs by older people. *J Am Geriatr Soc* 2002; **50**: 26–34.
9 Sloane PD, Zimmerman S, Brown LC, Ives TJ, Walsh JF. Inappropriate medication prescribing in residential care/assisted living facilities. *J Am Geriatr Soc* 2002; **50**: 1001–1011.
10 Graves T, Hanlon JT, Schmader KE *et al.* Adverse events after discontinuing medications in elderly outpatients. *Arch Intern Med* 1997; **157**: 2205–2210.
11 Hanlon JT, Weinberger M, Samsa GP *et al.* A randomized, controlled trial of a clinical pharmacist intervention to improve inappropriate prescribing in elderly outpatients with polypharmacy. *Am J Med* 1996; **100**: 428–437.
12 Hirakawa Y, Masuda Y, Uemura K *et al.* Current admission policies of long-term care facilities in Japan. *Geriatrics Gerontol Int* 2003; **3**: 73–78.
13 Naranjo CA, Busto U, Sellers EM *et al.* A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther* 1981; **30**: 239–245.
14 Stewart RB, Cooper JW. Polypharmacy in the aged. Practical solutions. *Drugs Aging* 1994; **4**: 449–461.
15 Linjakumpu T, Hartikainen S, Klaukka T, Veijola J, Kivela SL, Isoaho R. Use of medications and polypharmacy are increasing among the elderly. *J Clin Epidemiol* 2002; **55**: 809–817.
16 Martin I, Hall J, Gardner T. Prescribing for patients aged 65 years and over in New Zealand general practice. *N Z Med J* 2002; **115**: U221.
17 Koopmans RT, van der Borgh JP, Bor JH, Hekster YA. Increase in drug use after admission to Dutch nursing homes. *Pharm World Sci* 2003; **25**: 30–34.

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Caveolin-1, Id3a and two LIM protein genes are upregulated by estrogen in vascular smooth muscle cells

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Abstract

Estrogen has diverse effects on the vasculature, such as vasodilation, endothelial growth and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration. However, little is known about the genes that are regulated by estrogen in the vascular wall. Wistar rats were ovariectomized or sham-operated (Sham group), and 2 weeks after the operation, were subjected to subcutaneous implantation of placebo pellets (OVX + V group) or estradiol pellets (OVX + E group). Endothelium-denuded aortic tissue was examined 2 weeks after implantation. By applying high-density oligonucleotide microarray analysis, the expression of approximately 7000 genes was analyzed. Among the genes with different expression levels between the OVX + E group and the OVX + V group, those that have been reported to be expressed in the vasculature or muscle tissue, were chosen. Finally, four genes, caveolin-1, two LIM proteins (enigma and SmLIM) and Id3a, were identified. Microarray as well as real-time polymerase chain reaction showed that the expression levels of these genes were significantly higher in the OVX + E group than in the OVX + V group. To clarify whether estrogen directly upregulates these genes in the vascular wall, Northern blot analysis was performed using cultured rat VSMC. Addition of 100 nmol/L estradiol for 24 hours increased the mRNA levels of all four genes. Although the

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precise mechanism remains unclear, regulation of these genes by estrogen might contribute to its effect on VSMC.

Keywords: Atherosclerosis; Gene expression; Hormones; Smooth muscle

Introduction

Epidemiological studies have shown that the risk for cardiovascular disease is lower in premenopausal women than in men of the same age. Hormone replacement therapy has been reported to lower the incidence of cardiovascular disease in postmenopausal women (Colditz et al., 1987; Kannel et al., 1976), although the beneficial effects of estrogen have not been confirmed in recent randomized trials (Hulley et al., 1998; Rossouw et al., 2002). A number of animal studies have also shown estrogen's anti-atherogenic effects, including amelioration of the response to vascular injury (Sullivan et al., 1995), inhibition of endothelial cell apoptosis (Sudoh et al., 2001), and nitric oxide-mediated vasodilatation (Bell et al., 1995). Estrogen receptors (ER) are expressed in the vasculature (Hodges et al., 2000; Karas et al., 1994), supporting that estrogen can exert its effect directly on the vascular wall.

Several estrogen-responsive genes, such as pS2 (Brown et al., 1984), c-fos (Weisz and Bresciani, 1988), and efp (Inoue et al., 1993), have already been identified in reproductive tissues. In the vasculature, estrogen-regulated genes without estrogen-responsive elements in their promoter region are reported (Akishita et al., 1996; Gallagher et al., 1999; Nickenig et al., 1998). The expression of c-fos (Akishita et al., 1996), angiotensin-converting enzyme (Gallagher et al., 1999), and angiotensin receptor-1 (Nickenig et al., 1998) in the aorta was downregulated by estrogen replacement in ovarietomized rats. These changes of gene expression could explain a part of atheroprotective effects of estrogen. Recently, methods for global gene analysis have been developed, and among them, the high-density oligonucleotide microarray, has come to be used as a powerful tool by many investigators. In this study, to discover new genes that might play a role in the action of estrogen, we performed microarray analysis to identify genes that are differentially expressed in the vascular wall, especially in vascular smooth muscle cells (VSMC), before and after treatment with estrogen. To confirm the results obtained from the microarray, we performed real-time polymerase chain reaction (PCR) and Northern blotting. Finally, four genes were identified as novel estrogen-regulated genes in VSMC.

Methods

Animals

Eight-week-old female Wistar rats (Oriental Yeast, Co., Ltd., Tokyo, Japan) were used in this study. They were kept individually in stainless-steel cages in a room where lighting was controlled (12 hours on, 12 hours off) and room temperature was kept at around 22 °C. They were given a standard diet and water ad libitum. All the surgical procedures were performed under ether anesthesia. All of the experimental protocols were approved by the Animal Research Committee of the University of Tokyo.

Ovariectomy and E2 Implantation

Rats were randomly divided into three groups. Two groups of rats were ovariectomized and the other group of rats was sham-operated. After a two-week recovery period, one group of ovariectomized rats (OVX + E group, n = 5) underwent subcutaneous implantation of a three-week releasing pellet containing 0.5 mg 17β-estradiol (E2; Innovative Research of America). The other group of ovariectomized rats (OVX + V group, n = 5) and sham-operated rats (Sham group, n = 4) received placebo pellets. Two weeks after pellet implantation, blood samples were obtained from rats. Serum estradiol concentration was 5.6 ± 1.5 pg/ml in the Sham group (n = 4), 2.8 ± 1.0 pg/ml in the OVX + V group (n = 5), and 74.5 ± 12.1 pg/ml in the OVX + E group (n = 5). The thoracic aorta was obtained from rats after sacrifice. The endothelium was removed from the aorta by scraping with blade to ensure that the sample was mainly derived from VSMC.

High-density oligonucleotide microarray analysis

Total RNA was extracted from the aorta with Isogen (Wako Junyaku Ltd.) according to the manufacturer's instructions. One microgram of RNA isolated from the aorta of OVX + E group, OVX + V group and Sham group (n = 2, each group) rats was amplified up to approximately 100 μg cRNA and hybridized to the high-density oligonucleotide microarray (GeneChip Rat GenomeU34A; Affymetrix, Santa Clara, CA) as described previously (Ishii et al., 2000). This array contains probes interrogating approximately 7000 full-length rat genes. The intensity for each feature of the array was calculated by using Affymetrix Gene Chip version 3.3 software. The average intensity was made equal to the target intensity, which was set at 100, to reliably compare variable multiple arrays. In addition to the default parameters of the software, we added a criteria that >100 average intensity units per transcript was required for a gene to be considered "present" in the samples. Genes, with an intensity of around 1.5-fold higher or lower in the OVX + E group than in the OVX + V group, were identified.

Real-time PCR

Total RNA was treated with DNase (Progema) at 37°C for 1 h. One microgram of RNA was reverse transcribed into cDNA using Oligo dT primer (GIBCO) and an Ominiscript kit (GIBCO). Real-time PCR was carried out in an iCycler (BioRad) at 95°C for 15 min to activate HotStar Taq DNA polymerase, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec using a SYBR green assay kit (TAKARA). Amplicons were around 100 bp long. We selected the primer sets that amplified the sequences as close as possible to the 3′ coding region of the target genes. The sequences of the primers are shown in Table 1. The expression levels of each gene were normalized for glyceraldehyde-3-phosphate dehydrogenase expression.

Cell culture

VSMC were harvested from the aorta of Wistar rats by enzymatic dissociation, as previously reported (Watanabe et al., 2001). Cells were maintained in Dulbecco's modified Eagle's medium (Nikken Bio Medical Laboratory, Tokyo) supplemented with 10% fetal bovine serum (Intergen Co., Purchase, NY), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air and