by real-time PCR using an SYBR green dye. Primers used are listed in Table 1. Real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA).

Western blot analysis. Western blot analysis was performed as previously described (21). To measure the contents of Ang-1 in culture medium, the culture medium was concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA).

Chemotaxis assay. ASCs were cultured in EBM or EGM for 7 days, and the medium was changed to DMEM-0.2% FBS. The medium was collected after 12 h and used for chemotaxis assay. Chemotaxis assay was performed using a chemotaxis assay chamber according to the instructions provided by the manufacturer (Neuro Probe, Gaithersburg, MD). In brief, the medium collected from ASCs that contains chemoattractants was placed under the filter. HUVECs were suspended in DMEM-0.2% FBS at a density of 1×10^6 cells/ml, and 25 μl each of the cell suspension was placed on the filter. After 24 h, the upper side of the filter was scrubbed with a cotton swab and washed with PBS to scrape off cells attaching to the side. The lower side of the filter was fixed with 100% methanol, and the cells on the lower side were stained with hematoxylin. The cell number on the filter was counted in three random high-power fields (×100) in each well, and the average of the cell number was used for statistical analysis.

Adenoviral infection. A recombinant adenovirus that expresses green fluorescence protein (AdGFP) was obtained from Quantum Biotechnologies (Montreal, Canada). ASCs were infected with AdGFP at a multiplicity of infection of 40 and used for in vivo experiments.

Wire injury model. All procedures involving experimental animals were approved by the Institutional Committee for Animal Research of the Tokyo University. Transluminal mechanical injury to rat femoral artery was performed as previously described (18). Male Wistar rats (8 to 10 wk old) were anesthetized with pentobarbital sodium injected intraperitoneally, and a groin incision was made under a surgical microscope. A guide wire (0.46 mm diameter) was introduced through a small muscular branch of the femoral artery proximally to the aortic bifurcation and withdrawn. ASCs cultured in EBM or EGM (10^6 cells) for 7 days were injected into the femoral artery and incubated for 30 min with the proximal and distal sides of the artery clamped. In

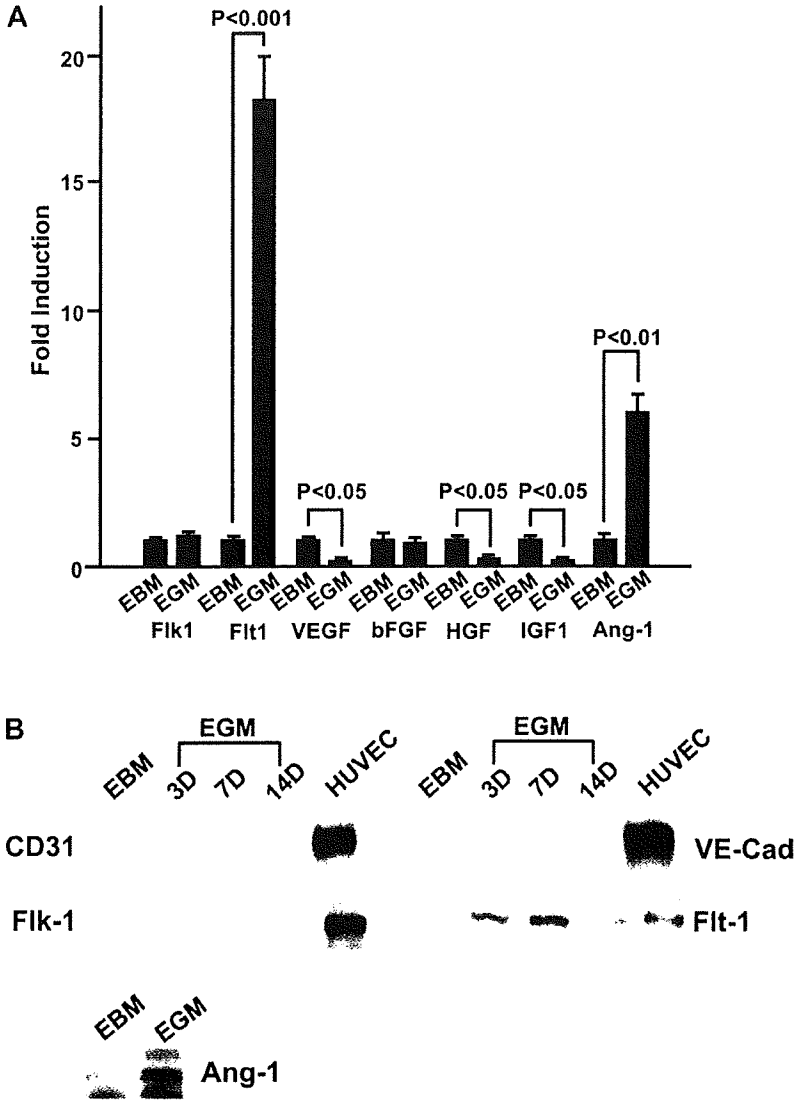

Fig. 2. *A*: real-time PCR analysis of mRNA expression in ASCs. ASCs were cultured in endothelial basal medium (EBM) or endothelial growth medium (EGM) for 7 days, and total RNA was extracted for real-time PCR analysis. Expression of GAPDH was used as the internal control ($n = 5$ experiments). VEGF, VEGF-A; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor. *B*: expression of endothelial cell-specific markers and production of angiopoietin-1 (Ang-1) in ASCs. ASCs were cultured in EBM for 7 days or in EGM for the indicated periods. Protein extracts were used for Western blot analysis. To detect Ang-1 in culture medium, ASCs were cultured in EBM or EGM for 7 days, and the medium was replaced with DMEM-0.2% FBS. The medium was harvested after 12 h and concentrated for Western blot analysis. Shown are representative results of 3 independent experiments. HUVEC, human umbilical vein endothelial cell; 3D, 3 days; 7D, 7 days; 14D, 14 days.

some experiments. ASC suspension was dropped around the femoral artery from the adventitial side after wire injury.

Histochemistry. The femoral arteries were fixed by perfusing them with 4% paraformaldehyde and processed for paraffin embedding. Cross sections (2 μm) were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin. For immunohistochemistry, the sections were incubated with primary antibodies reactive to PCNA and CD31. The sections were then incubated with biotinylated secondary antibody and finally horseradish peroxidase-labeled streptavidin according to the instructions provided by the manufacturer (DAKO). The sections were counterstained with hematoxylin.

Statistical analysis. Values are means ± SE. Statistical analyses were performed using analysis of variance followed by the Student-Newman-Keuls test. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

Characterization of ASCs. Cell surface markers were first analyzed using flowcytometry analysis (Fig. 1). In contrast to previous reports showing that ASCs isolated from humans expressed CD34 to some extent (9, 15, 16), CD34 expression was negative in ASCs derived from Wistar rats. The expression of VE-cadherin was also negative, suggesting that the contamination of ECs was negligible. The expression of CD29 and CD90 was positive. These cell surface markers were reportedly positive in BMMSCs (14). Therefore, our results suggested that ASCs isolated from Wistar rats resembled mesenchymal stem cells rather than hematopoietic stem cells.

Expression patterns of mRNA and protein in ASCs. We cultured ASCs on fibronectin-coated dishes and examined mRNA expression in ASCs cultured in either EBM or EGM (Fig. 2*A*). The expression of Flk-1 was not significantly induced in ASCs cultured in EGM compared with those cultured in EBM. The expression of mature EC markers such as CD31 and VE-cadherin was significantly suppressed when ASCs were cultured in EGM (data not shown). In contrast, the expression of Flt-1 significantly increased in ASCs cultured in EGM compared with those cultured in EBM. We also examined the mRNA expression of proangiogenic factors and anti-apoptotic factors that ASCs might secrete. The expression of VEGF-A and IGF-1 significantly decreased in ASCs cultured in EGM compared with those cultured in EBM, probably because EGM contains VEGF-A and IGF-1 to induce the differentiation into ECs. The expression of bFGF was not significantly changed between EBM-cultured ASCs and EGM-cultured ASCs. The expression of HGF was significantly suppressed in ASCs cultured in EGM. In contrast, the expression of Ang-1 significantly increased in ASCs cultured in EGM compared with those cultured in EBM. We next examined the expression level of some of these genes at the protein level (Fig. 2*B*). The expression of Flk-1, CD31, or VE-cadherin was not detected in ASCs cultured in EGM until up to 14 days after incubation with EGM. In contrast, the expression of Flt-1 was detected 3 days after incubation with EGM and peaked 7 days after culture in EGM. The ASCs cultured in EGM also secreted a higher amount of Ang-1 into the culture medium than EBM-cultured ASCs. Since Flt-1 is expressed in monocytes/macrophages as well as ECs (20), the expression of CD14 was examined by Western blot analysis and immunostaining, but its expression was not detected in ASCs cultured in EGM (data not shown), suggesting that the contamination of monocytes/macrophages was negligible. Collectively, our data suggested that ASCs do not appear to have the potential to differentiate into mature ECs in vitro, although ASCs express Flt-1. Our results also suggested that ASCs might have the capacity to promote neovessel formation via a stimulation of the recruitment of ECs in situ, because ASCs, especially cultured in EGM, produced a significant amount of Ang-1.

ASCs stimulate migration of HUVECs. We, therefore, examined whether ASCs would stimulate the migration of ECs by chemotaxis assay (Fig. 3). Ang-1 was used as the positive control for the chemotaxis assay. Ang-1 (10 ng/ml) significantly stimulated the migration of HUVECs, and this effect was significantly suppressed when Ang-1 was preincubated with anti-Ang-1 antibody. Culture medium harvested from EGM-cultured ASCs significantly stimulated the migration of HUVECs compared with that harvested from EBM-cultured ASCs. This stimulatory effect was partially but significantly blocked by the preincubation of the culture medium with anti-Ang-1 antibody, suggesting that Ang-1 was, at least partly, responsible for the migration-stimulating effect.

ASCs inhibit neointimal formation via stimulation of endothelial repair in a paracrine fashion. We next examined the function of ASCs in vivo using the wire injury model of the rat femoral artery. When injected in the femoral artery, EBM-cultured ASCs slightly but significantly inhibited neointimal formation compared with wire injury without cell administration. EGM-cultured ASCs potently and more significantly inhibited neointimal formation compared with EBM-cultured ASCs (Fig. 4*A*). In accordance with these results, the number of PCNA-positive cells in the neointima was significantly suppressed in the group that was administered EBM-cultured ASCs compared with the group that received no cells. The number of PCNA-positive cells in the

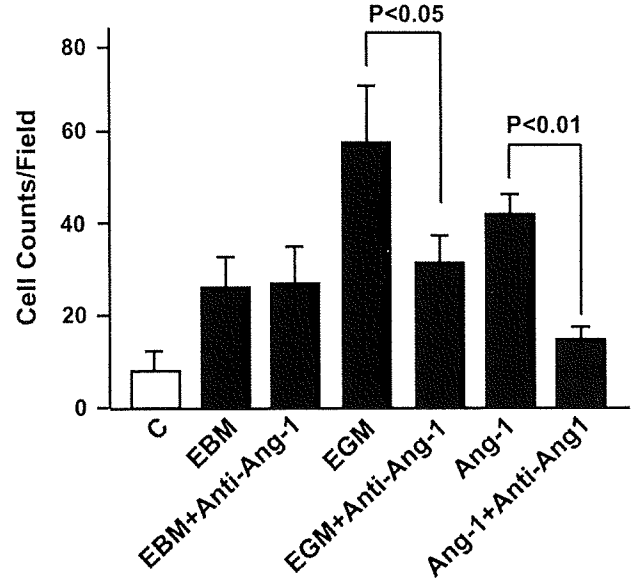

Fig. 3. ASCs produce paracrine factors that stimulate migration of HUVECs. ASCs were cultured in EBM or EGM for 7 days, and the medium was replaced with DMEM-0.2% FBS. The medium was collected after 12 h and used for chemotaxis assay. The conditioned medium was also preincubated with anti-Ang-1 antibody to neutralize Ang-1. Chemotaxis assays were performed as described in MATERIALS AND METHODS (n = 6 experiments). Human Ang-1 (10 ng/ml) was used as the positive control for the chemotaxis analysis.

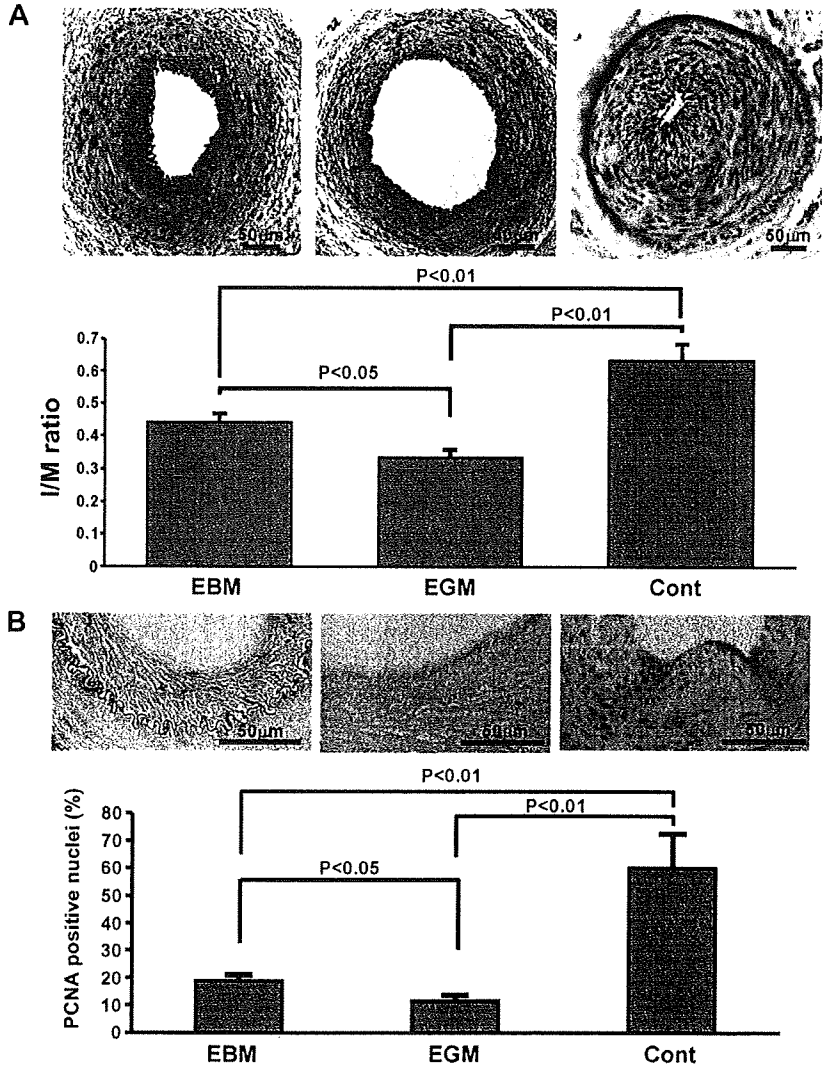

Fig. 4. ASC administration inhibits neointimal formation. *A*: ASCs cultured in EGM or EBM for 7 days (10^6 cells) were injected into the femoral artery immediately after wire injury, and the femoral arteries were harvested 14 days after the injury for histological analysis. Wire injured femoral artery without cell injection was also harvested and analyzed as the positive control (Cont). The ratio of the intimal to medial area (I/M) was compared between the groups (n = 8 experiments). *B*: proliferating cell nuclear antigen (PCNA)-positive cells in the neointima significantly decrease by administration of ASCs. Experiments were performed as described in *A*. The number of PCNA-positive cells was compared between the groups (n = 6 experiments).

neointima was more significantly reduced in the group that was administered EGM-cultured ASCs compared with the group that was administered EBM-cultured ASCs (Fig. 4*B*). Because ASCs cultured in EGM potently inhibited neointimal formation, we studied the mechanism whereby these cells inhibited neointimal formation. We examined whether EGM-cultured ASCs were engrafted into the endothelial layer and contributed to the repair of the endothelial layer after the wire injury. EGM-cultured ASCs were infected with AdGFP before injection into the artery. One day after injection, EGM-cultured ASCs were detected in the endothelial layer. However, EGM-cultured ASCs were barely detected in the endothelial layer 3 and 14 days after injection (Fig. 5*A*), suggesting that EGM-cultured ASCs inhibited neointimal formation without integrating into the endothelial layer. To confirm the specificity of the green fluorescence detected in the endothelial layer, we also injected ASCs without AdGFP infection and examined autofluorescence of the femoral artery (Fig. 5*B*). Green fluorescence was not detected in the endothelial layer in this case, suggesting that the green fluorescence detected in the endothelial layer was derived from AdGFP-infected ASCs that stayed in the endothelial layer. We, therefore, hypothesized that EGM-cultured ASCs potently inhibited neointimal formation by secreting paracrine factors that stimulate the repair of the endothelial layer, because we found that these cells produce a significant amount of Ang-1. To examine this possibility, we next administered EBM- and EGM-cultured ASCs from the adventitial side of the femoral artery after wire injury (Fig. 5*C*). Interestingly, EGM-cultured ASCs more significantly inhibited neointimal formation compared with EBM-cultured ASCs even when these cells were administered from the adventitial side. To further examine the role of paracrine factors secreted by ASCs, we originally tried to knock down endogenous Ang-1 production using small interfering RNA technology. However, the transfection efficiency of small interfering RNA by lipofection into rat ASCs was <10%, making it very difficult to examine the effect of gene knockdown in ASCs. We instead used rat VSMCs that also produce Ang-1 and NRK-52E cells that barely produce Ang-1. Rat VSMCs produced ~50% of Ang-1 mRNA com-

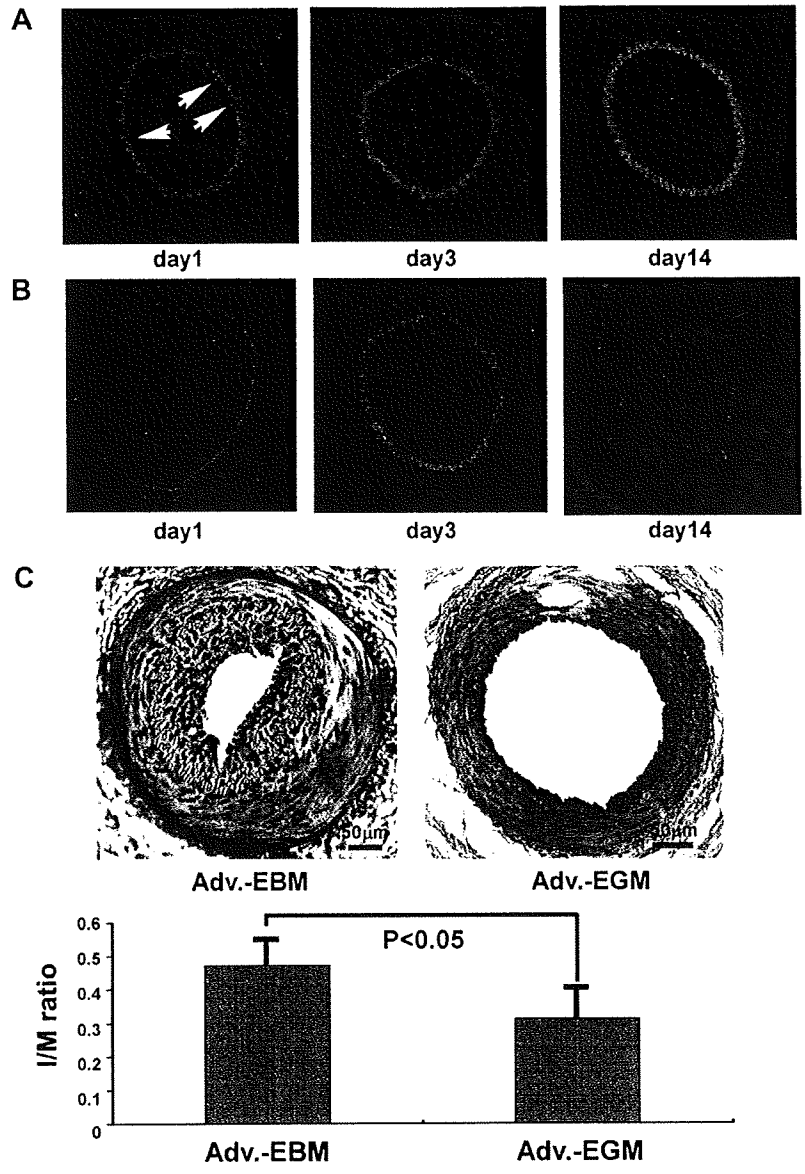

Fig. 5. *A*: time course of ASCs that remain in the endothelial layer. EGM-cultured ASCs were infected with adenovirus expressing green fluorescent protein (AdGFP) (40 multiplicity of infection) before injection into the femoral artery. Fluorescence of GFP was examined at the time points indicated. *B*: analysis of autofluorescence of the femoral artery. EGM-cultured ASCs were injected in the femoral artery without AdGFP infection, and autofluorescence of the femoral artery was examined at the time points indicated. *C*: ASCs administered from the adventitial side also significantly inhibit neointimal formation. ASCs cultured in EGM or EBM for 7 days (10^6 cells) were injected from the adventitial side (Adv.) of the femoral artery immediately after the wire injury. Femoral arteries were harvested 14 days after wire injury, and I/M ratio was compared between the groups (n = 6 experiments).

pared with ASCs cultured in EBM, and NRK-52E cells produced <1% of Ang-1 mRNA compared with ASCs cultured in EBM, as assessed by real-time PCR (data not shown). When rat VSMCs were administered from the adventitial side, they slightly but significantly inhibited neointimal formation compared with the NRK-52E cells administration (Fig. 6). These results also suggested that Ang-1 produced by ASCs might be, at least in part, implicated in the suppression of neointimal formation. We finally examined whether ASC administration from the adventitial side would stimulate the repair of the endothelial layer (Fig. 7). We examined the ratio of the endothelial layer positively stained with CD31. Endothelial repair was significantly enhanced by the administration of EGM-cultured ASCs compared with administration of EBM-cultured ASCs.

DISCUSSION

In this study, we isolated ASCs from Wistar rats and examined their characteristics in vitro and in vivo. ASCs obtained from Wistar rats expressed CD29 and CD90 but not CD34, suggesting that the ASCs we used resembled BMMSCs rather than hematopoietic stem cells. Although several studies demonstrated that human ASCs contain a large population of cells that express CD34 and that these CD34-positive cells differentiate into endothelial-like cells in vitro and in vivo (9, 15), ASCs do not always express CD34 in mice (6, 11). Although we do not know the reason for this discrepancy, cell surface markers of ASCs may differ among species.

ASCs used in this study expressed Flt-1 when they were cultured in EGM. However, ASCs did not express Flk-1 or

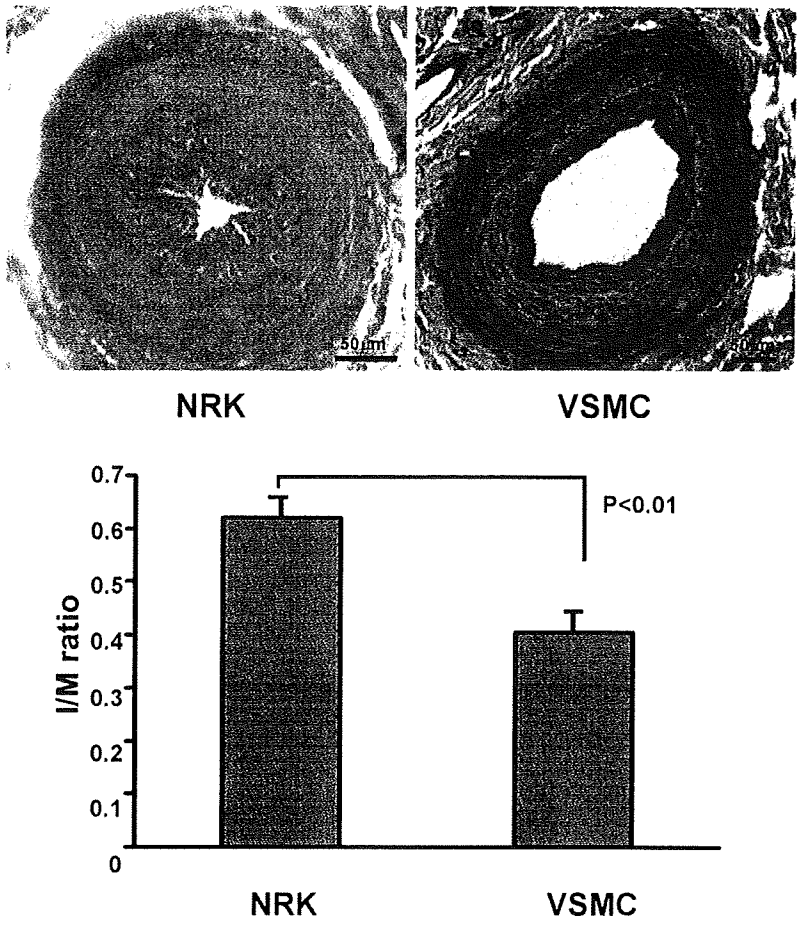

Fig. 6. Effects of administration of rat vascular smooth muscle cells (VSMCs) and NRK-52E cells (NRK) on neointimal formation. These cells were administered from the adventitial side of the femoral artery immediately after wire injury. Femoral arteries were harvested 14 days after wire injury, and I/M ratio was compared between the groups (n = 6 experiments).

mature EC markers such as VE-cadherin and CD31. Thus ASCs used in this study did not appear to have the capacity to differentiate into mature ECs. ASCs may resemble bone marrow-derived cells that express Flt-1 and are recruited to sites of ischemia (7, 12). Although several studies showed that human ASCs had the potential to differentiate toward ECs in vitro by demonstrating the expression of endothelial markers such as CD31, the efficiency varies so much, probably because the methodology whereby they induced differentiation of ASCs into ECs differs from study to study (3, 8, 9, 15). One study demonstrated that mouse ASCs could differentiate into ECs in vitro by examining the expression of CD31 and VE-cadherin, but its efficiency seemed to be very low (11). Recently, Boquest et al. (2) examined the methylation profiles of EC-specific gene promoters such as CD31 and VE-cadherin and showed that the promoters of CD31 and VE-cadherin were hypermethylated in ASCs and that these promoters seemed to have a relatively small potential to be activated in ASCs. Moreover, whether ASCs can differentiate into ECs in vivo remains debatable. Several studies demonstrated that ASCs were integrated into capillaries in hindlimb ischemia models and improved blood flow via the stimulation of angiogenesis (9, 15). However, ASCs could reportedly stimulate angiogenesis and restore blood flow in hindlimb ischemia models without being engrafted into capillaries, probably because of their paracrine effects (11). Therefore, the efficiency of ASCs to differentiate into ECs in vitro and in vivo appears to differ, depending on the cell culture conditions, animal model used, and animal species. Future studies will be required to elucidate an appropriate strategy to efficiently induce differentiation of ASCs into mature ECs.

We, therefore, examined paracrine factors that ASCs produce. ASCs reportedly produce a variety of proangiogenic and antiapoptotic factors such as VEGF, IGF, HGF, and bFGF. Although the production of these factors did not increase when ASCs were cultured in EGM, the production of Ang-1 was significantly increased. Furthermore, ASCs appeared to secrete functionally active Ang-1, as assessed by chemotaxis assay using HUVECs. These results suggested that ASCs potentially promote repair of the endothelial layer via stimulation of migration of ECs in situ.

To test this hypothesis, we administered ASCs in a wire injury model of rat femoral artery. EGM-treated ASCs significantly inhibited neointimal formation without being engrafted into the endothelial layer. EGM-treated ASCs also significantly suppressed neointimal formation even when they were administered from the adventitial side. Endothelial repair occurred more rapidly in rats administered EGM-cultured ASCs compared with those administered EBM-cultured ASCs. The rapid endothelial repair was accompanied by less cell proliferation in

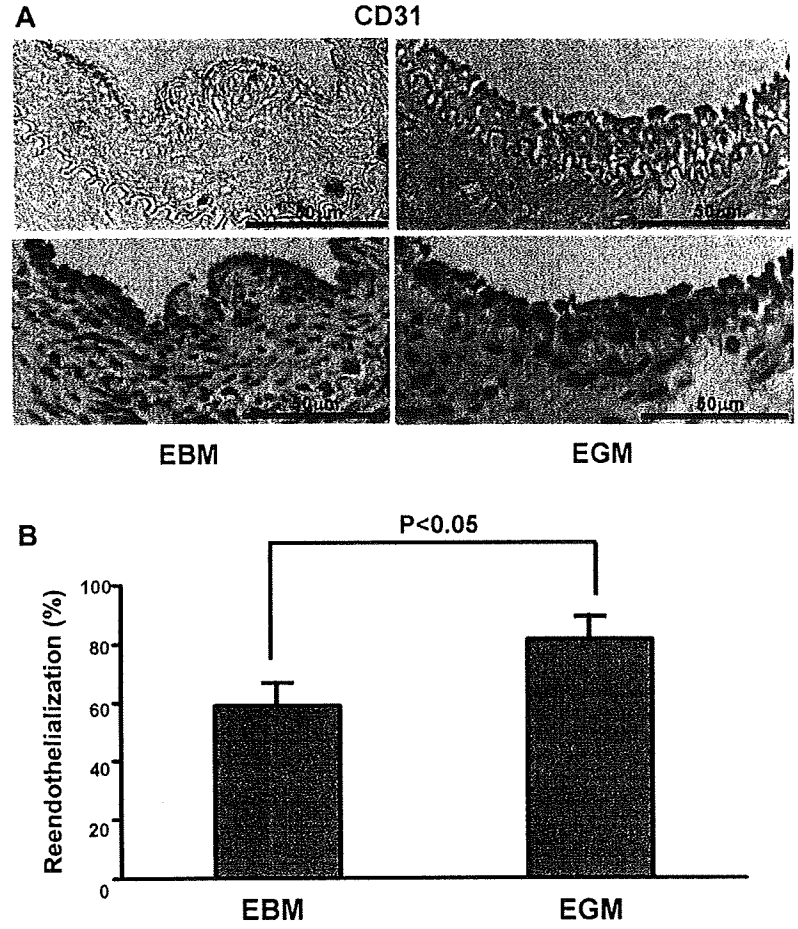

Fig. 7. Administration of EGM-cultured ASCs promotes endothelial repair. *A*: EBM- or EGM-cultured ASCs were administered from the adventitial side after the wire injury. Femoral arteries were harvested 14 days after the injury, and CD31 was stained to detect repaired endothelial layer (*top*). The specimens were next counterstained with hematoxylin to show the nuclei of the endothelial cells (*bottom*). *B*: ratio of the CD31-positive reendothelialized area (n = 6 experiments).

the neointima. Furthermore, the administration of VSMCs that also produce Ang-1 slightly but significantly suppressed neointimal formation, whereas the administration of NRK-52E cells that barely produce Ang-1 did not. These results suggested that ASCs inhibited neointimal formation in a paracrine fashion via the stimulation of endothelial repair. These results also suggested that Ang-1 was, at least in part, implicated in the inhibitory effect of ASCs on neointimal formation. It has been reported that EPCs, when injected in the carotid artery, were engrafted in the endothelial layer in a balloon injury model. However, it remains unclear how long the injected EPCs can survive and proliferate in the endothelial layer. EPCs were not detected in the endothelial layer 30 days after the administration in that study (5). Thus, although EPCs seem to be more effectively integrated in the endothelial layer than ASCs, EPCs may also stimulate endothelial repair via a stimulation of EC migration in situ, because EPCs also reportedly produce paracrine factors such as VEGF (23). These possibilities should be addressed in the future.

In summary, although rat ASCs do not differentiate into mature ECs, they produce paracrine factors such as Ang-1, especially when they were cultured in EGM. These factors seem to stimulate the migration of ECs in situ and the repair of the endothelial layer in vivo. Although the capacity of ASCs to differentiate into mature ECs may be low, ASCs will be useful for cell-based therapy to treat cardiovascular diseases such as hindlimb ischemia, acute myocardial infarction, and the prevention of restenosis after angioplasty via their capacity to produce paracrine factors that stimulate angiogenesis and endothelial repair.

GRANTS

This study was supported in part by research Grants from Japan Science Technology Agency (Core Research for Evolutionary Science and Technology) (to T. Nagano and Y. Hirata).

DISCLOSURES

There exist no conflicts of interest.

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Official Journal of the Japanese Circulation Society
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Relationship Between Renal Dysfunction and Severity of Coronary Artery Disease in Japanese Patients

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Background: The relationship between renal dysfunction and the severity of coronary artery disease (CAD) was examined.

Methods and Results: The severity of CAD in 572 patients was graded according to the number of stenotic coronary arteries, and the estimated glomerular filtration rate (eGFR) was monitored for 3 years. Patients were stratified into 3 eGFR groups: normal (>75 ml·min^{-1}·1.73 m^{-2}), mild reduction (60–75) and chronic kidney disease (CKD: <60). There were 161 patients in the CKD group. The average number of stenotic coronary arteries was larger in the CKD group than in the other groups (normal vs mild reduction vs CKD=1.35±0.07 (SE) vs 1.22±0.08 vs 1.69±0.08 vessel disease (VD), P<0.001). During the 3-year follow-up, the renal function of 13.8% of the patients worsened. Those who showed more deterioration of eGFR had more severe CAD than those who did not (1.20±0.06 vs 1.61±0.06 VD, P<0.001). Multivariate analysis revealed that the severity of CAD was independently and significantly associated with the deterioration of eGFR.

Conclusions: Patients with CKD had more severe CAD, which may explain the high rate of cardiovascular events in these patients. Moreover, the prognosis of renal function was poor in patients with severe CAD, and CAD was found to be an independent risk factor for worsening of renal dysfunction.

Key Words: Chronic kidney disease; Coronary artery disease; Glomerular filtration rate; Renal function

It is well established that decreased renal function is associated with an increased frequency of cardiovascular disease, so patients with end-stage renal disease have a very high risk for cardiovascular events. However, this is the case even in patients with mildly reduced renal function. In fact, Go et al reported that among the American population patients with mild chronic kidney disease (CKD), such as those whose glomerular filtration rate (GFR) is between 45 and 59 ml·min^{-1}·1.73 m^{-2}, already showed substantial increases in the frequency of cardiovascular events.[1] This has been confirmed not only in population-based epidemiological studies, but also in clinical trials.[2–6] However, the mechanisms of the involvement of renal dysfunction in the occurrence of cardiovascular events remain unclear, although several possibilities have been suggested. It is also unclear which cardiovascular events are likely to occur in patients with renal damage. According to previous reports, coronary artery disease (CAD), including acute myocardial infarction (AMI), is the most frequent type of cardiovascular event in patients with CKD.[3–6] Furthermore, the prognosis of such patients is worse than in those without CKD.[1,2] A Japanese population study recently showed that the risk of cardiovascular events increased as renal function decreased.[7] In that study the leading etiology of the cardiovascular events was cerebral vascular accidents rather than CAD. In Asian countries, particularly in Japan, the occurrence of stroke is twice that of CAD.[8] Nonetheless, the prevalence of AMI is higher in patients with decreased renal function.[7]

In the present study, we explored the relationship between renal dysfunction and the severity of CAD by counting the number of stenotic coronary arteries in Japanese patients. Furthermore, as cardiovascular damage has been suggested to aggravate renal dysfunction,[9–11] we followed patients with CAD for 3 years to examine the influence of CAD on renal function.

Methods

For this study, data from 572 consecutive Japanese patients who underwent scheduled coronary angiography (CAG) at the University of Tokyo Hospital under the suspected diagnosis of CAD from August 1999 to February 2004 were analyzed retrospectively.

Scheduled CAG was performed using a transradial, trans-

Received September 28, 2009; accepted December 14, 2009; released online February 17, 2010 Time for primary review: 36 days
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ISSN-1346-9843 doi:10.1253/circj.CJ-09-0715

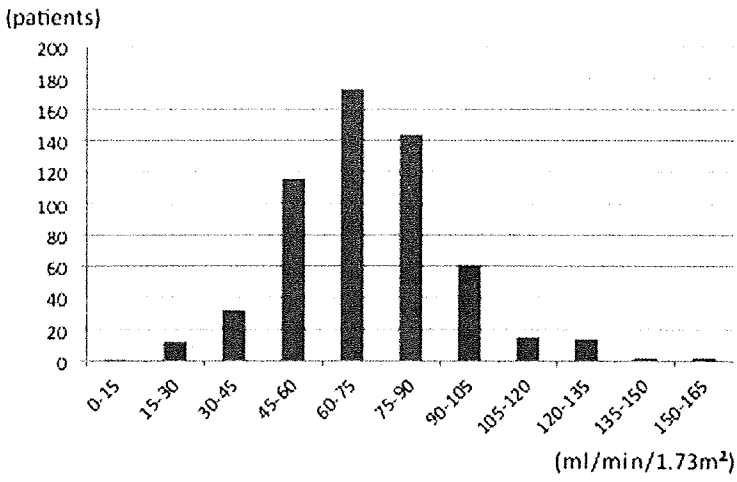

Figure 1. Distribution of the estimated glomerular filtration rate.

Table 1. Clinical Profile of the Patients in Each Renal Function Group

	Total (eGFR)	Normal (>75)	Mild reduction (60–75)	CKD (<60)	P value ($ml \cdot min^{-1} \cdot 1.73 m^{-2}$)
n	572	238	173	161	
Sex, M/F	412/160	174/64	127/46	111/50	0.589
Age (years)	66.4±0.4	63.1±0.6	67.1±0.7	70.7±0.7	<0.001
BMI	23.9±0.1	24.0±0.2	23.5±0.2	24.2±0.3	0.151
Coexisting coronary risk factors					
Hypertension, %	92.7	91.6	92.5	94.4	0.570
Diastolic BP, mmHg	77.0±12.9	77.5±13.5	77.8±13.1	75.4±11.7	0.178
Systolic BP, mmHg	135.6±20.7	134.9±20.5	135.9±20.9	136.2±20.9	0.824
Diabetes mellitus, %	36.1	37.0	35.0	36.0	0.852
Dyslipidemia, %	63.1	63.4	64.2	61.0	0.872
Smoking habit, %	61.2	61.3	62.4	60.0	0.870
No. of coronary risk factors/patient	2.53±0.04	2.54±0.06	2.54±0.07	2.52±0.07	0.967
Serum Cr, mg/dl	0.84±0.01	0.64±0.01	0.82±0.01	1.14±0.03	<0.001
eGFR, $ml \cdot min^{-1} \cdot 1.73 m^{-2}$	72.1±0.9	91.1±1.0	68.0±0.3	48.5±0.8	<0.001
LVEF, %	66.0±12.1	66.4±12.2	66.7±12.1	64.7±11.8	0.294
Plasma BNP, pg/ml	85±205	67±142	59±92	140±328	<0.001

Data are mean±SE or percentage.
eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; BMI, body mass index; BP, blood pressure; Cr, creatinine; LVEF, left ventricular ejection fraction; BNP, B-type natriuretic peptide.

brachial or transfemoral approach. All angiographic reports were reviewed by at least 2 operators. The severity of coronary artery stenosis was assessed in the worst view projection and the percentage of luminal narrowing was recorded according to the American Heart Association criteria.[12] Luminal narrowing >51% was considered as significant stenosis. The left anterior descending, left circumflex and right coronary arteries were evaluated, and the number of stenotic arteries was recorded (0 to 3-vessel disease (VD)). A significant stenosis in the left main trunk was scored as 2VD.

Patients were admitted 1–3 days before the day CAG was to be performed. Body weight and blood pressure were measured in the morning of the day of admission. Blood samples were obtained from the antecubital vein, while the patient was supine, in the morning after an overnight fast. The plasma B-type natriuretic peptide (BNP) concentration was measured by enzymatic immunoassay,[13] and that of serum creatinine (Cr) by an enzymatic method using a standard autoanalyzer. Echocardiographic parameters were measured within 1 month after diagnostic CAG. The left ventricular (LV) dimension was measured on the long-axis view of the left ventricle taken with the patient in the left lateral position. LV ejection fraction (LVEF) was obtained by the following formula: LVEF=(LV end-diastolic volume−end-systolic volume)/LV end-diastolic volume. To evaluate cardiovascular risk factors, the numbers of smokers and patients with hypertension, diabetes mellitus or dyslipidemia were determined. Hypertension was defined as blood pressure >140/90 mmHg or use of antihypertensive agents; diabetes mellitus by fasting blood glucose ≥126 mg/dl or use of hypoglycemic agents or insulin; dyslipidemia by low-density lipoprotein cholesterol level ≥140 mg/dl, high-density lipoprotein cholesterol level ≤40 mg/dl or use of lipid lowering agents; and smoking by present or past smoking.

The patients were divided into 3 groups according to their estimated GFR (eGFR) calculated by the Modification of

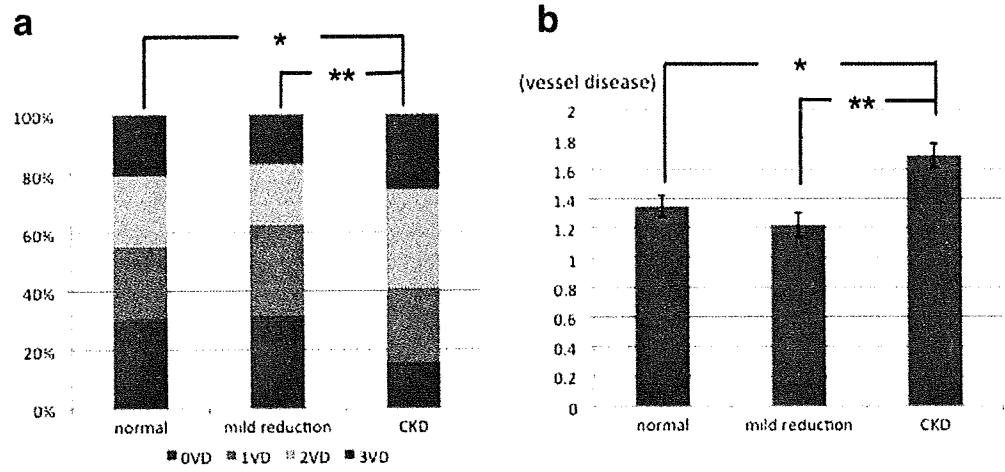

Figure 2. (a) Percentages of patients with 0 to 3-vessel disease (VD) in each group. (b) Number of stenotic coronary arteries in each group. Each column represents the mean±SE. The percentages of patients with multivessel stenosis (2VD+3VD) were significantly greater in the chronic kidney disease (CKD) group (*P<0.01, **P<0.001).

Table 2. Clinical Background of Patients Whose Renal Function Remained Unchanged (Upper Half) or Decreased (Lower Half) During Follow-up

	Total	Upper half	Lower half	P value
n	572	286	286	
Age, (years)	66.4±0.4	65.5±0.6	67.3±0.5	0.020
Sex, M/F	412/160	211/75	201/85	0.352
BMI	23.9±0.1	24.0±0.2	23.8±0.2	0.599
Coexisting coronary risk factors				
Hypertension, %	92.7	91.3	94.1	0.200
Systolic BP, mmHg	135.6±0.9	131.6±1.1	139.5±1.3	<0.001
Diastolic BP, mmHg	77.0±0.5	76.0±0.7	78.0±0.8	0.076
Diabetes mellitus, %	36.2	31.1	41.3	0.012
Dyslipidemia, %	63.1	62.2	64.0	0.665
Smoking habit, %	61.2	58.4	64.0	0.170
No. of coronary risk factors/patient	2.53±0.04	2.43±0.05	2.63±0.05	0.008
Baseline serum Cr, mg/dl	0.84±0.01	0.85±0.01	0.82±0.02	0.253
Serum Cr after 3 years, mg/dl	1.01±0.03	0.83±0.01	1.19±0.02	0.001
Baseline GFR, ml·min^{-1}·1.73 m^{-2}	72.1±0.9	69.5±1.0	74.6±1.4	0.003
GFR after 3 years, ml·min^{-1}·1.73 m^{-2}	62.4±0.8	69.9±1.0	54.9±1.2	<0.001
Change in GFR after follow-up, %	−12.8±0.8	1.4±0.7	−27.0±0.8	<0.001
LVEF, %	66.0±0.5	66.4±0.7	65.3±0.7	0.271
Plasma BNP, pg/ml	85.1±8.6	69.3±8.5	100.1±14.7	<0.001
Administration of ACEI or ARB, %	49.8	48.9	51.7	0.358
No. of stenotic coronary arteries, VD	1.41±0.05	1.20±0.06	1.61±0.06	<0.001
No. of CAG and PCI/patient during follow-up	2.81±0.09	2.57±0.12	3.05±0.14	0.012

Data are mean±SE or percentage.
GFR, glomerular filtration rate; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; VD, vessel disease; CAG, coronary angiography; PCI, percutaneous coronary intervention. Other abbreviations see in Table 1.

Diet in Renal Disease (MDRD) equation[14] with coefficients modified for Japanese patients:[15] eGFR (ml·min^{-1}·1.73 m^{-2})= 194×Cr$^{-1.094}$×age$^{-0.287}$ (×0.739 if female). The normal group had an eGFR >75 ml·min^{-1}·1.73 m^{-2}; the mild reduction group had an eGFR between 60 and 75 ml·min^{-1}·1.73 m^{-2}; the CKD group had an eGFR <60 ml·min^{-1}·1.73 m^{-2}. The patients were excluded because of unstable renal function if they had overt congestive heart failure or AMI, or were on hemodialysis.

The study was approved by the institutional ethical committee (No. 2252).

All study subjects visited hospital regularly as outpatients after discharge. eGFR was monitored for 3 years after diagnostic CAG. To evaluate the effect of contrast media admin-

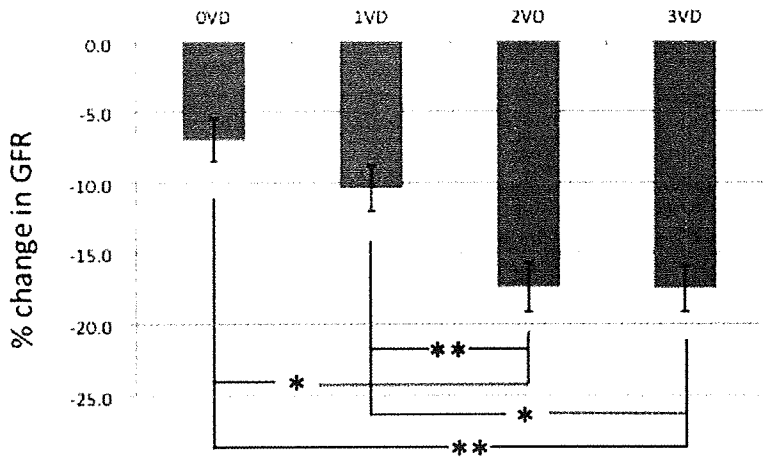

Figure 3. Percent glomerular filtration rate (GFR) deterioration during follow-up in patients with 0 to 3-vessel disease (VD). Each column represents the mean±SE. *P<0.01, **P<0.001.

Table 3. Correlation Between the Percent GFR Deterioration During Follow-up and Clinical Parameters

	Univariate analysis		Multivariate analysis (r^2=0.1417)		
	r^2	P value	β	F value	P value
Age	0.0031	0.1828	0.0739	1.5479	0.1223
Sex	0.0041	0.1244	–	–	–
BMI	0.0001	0.8558	–	–	–
Coexisting coronary risk factors					
Hypertension	0.0001	0.7903	–0.0539	–1.2641	0.2068
Systolic BP	0.0351	<0.0001	0.1542	2.9390	0.0034
Diastolic BP	0.0069	0.0488	0.0265	0.5088	0.6111
Diabetes mellitus	0.0209	0.0005	0.0875	2.0253	0.0434
Hyperlipidemia	0.0021	0.2795	0.0395	0.9055	0.3656
Smoking habit	0.0021	0.2736	–0.0068	–0.1638	0.8700
Baseline eGFR	0.0135	0.0053	0.1812	4.0747	0.0001
LVEF	0.0045	0.1092	–0.0157	–0.3480	0.7280
Plasma BNP	0.0468	<0.0001	0.1953	4.2209	<0.0001
No. of stenotic coronary arteries	0.0399	<0.0001	0.1140	2.4282	0.0155
No. of CAG and PCI/patient during follow-up	0.0118	0.0094	0.0379	0.8365	0.4033

Abbreviations see in Tables 1, 2.

istered during CAG or percutaneous coronary intervention (PCI), the frequency of exposure of each patient to contrast media during those 3 years was recorded.

Statistical Analysis

Values are expressed as the mean±SE. Statistical analyses were performed using SPSS version 17.0 (Chicago, IL, USA). Unpaired Student's t-test was used for comparisons between 2 groups. Tukey's multiple comparison of means following ANOVA was used for comparisons among more than 2 groups. A multiple linear regression analysis of independent predictors of renal prognosis was also performed. The level of statistical significance was set at P<0.05.

Results

The baseline eGFR of the 572 patients showed a normal distribution (**Figure 1**), and the mean was 72.1±0.9 ml·min^{-1}·1.73 m^{-2} (median, 71.7; interquartile range, 58.0–84.7). There were 173 (30.2%) patients with a normal eGFR, 238 (41.6%) with a mildly reduced eGFR, and 161 (28.1%) with CKD. The clinical profile of the patients in each group is shown in **Table 1**. Although CKD patients were slightly older than those in the other groups and had decreased renal function, the prevalence of risk factors for CAD was similar among the 3 groups.

As for the severity of CAD, 151 patients (26.4%) had 1VD, 145 (25.3%) had 2VD and 123 (21.5%) had 3VD (**Figure 2a**). No significant stenotic lesions were detected in 153 (26.3%) patients. The percentages of patients with multivessel stenosis (2VD+3VD) were significantly greater in the CKD group (P<0.001). The CKD group had a significantly higher number of stenotic coronary arteries then the normal and the mild reduction groups (**Figure 2b**). Although blood pressure and LVEF did not differ significantly among the 3 groups, the CKD group had a significantly higher plasma level of BNP than the other 2 groups (**Table 1**).

At the end of the 3-year follow-up eGFR had decreased from 72.1±0.9 to 62.4±0.8 ml·min^{-1}·1.73 m^{-2} (median, 63.4; interquartile range, 50.1–73.9; P<0.001). The rate of decline was 3.2±0.2 ml·min^{-1}·1.73 m^{-2}·year^{-1} and showed a normal distribution; 79 (13.8%) patients were newly diagnosed with

CKD during the follow up. On the other hand, no CKD patient showed improvement of eGFR during the same period.

We examined the factors related to the deterioration of renal function. Table 2 compares the clinical background of patients with unchanged renal function (ie, patients included in the upper half of the percent deterioration of eGFR) with that of patients with worsened renal dysfunction (the lower half). Age, systolic blood pressure, prevalence of diabetes mellitus, baseline eGFR, plasma BNP, number of coronary risk factors and number of CAG and PCI during follow-up per patient were found to be significantly higher in the lower half group (ie, the group showing a greater reduction of eGFR). There was no significant difference in the medications nor in the frequency of administration of angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ARB) between the 2 groups. The number of stenotic coronary arteries was significantly greater in patients with decreased renal dysfunction compared with patients with unchanged renal function. The percent eGFR deterioration during follow up was significantly higher in the patients whose diagnostic CAG revealed multivessel disease (Figure 3). Stepwise multiple regression analysis was performed to evaluate the independent determinants of the percent eGFR deterioration (Table 3) and it was found that the number of stenotic coronary arteries, systolic blood pressure, prevalence of diabetes mellitus, baseline eGFR and plasma BNP, but not the number of CAG and PCI, showed an independent and significant association with the percent eGFR deterioration during follow-up.

Discussion

The incidence of cardiovascular disease increases in patients with reduced renal function. Although the exact mechanisms by which impaired renal function relates to cardiovascular disease remain unclear, many possibilities have been suggested; for example, renal dysfunction activates the renin–angiotensin system and sympathetic nervous system, elevates blood pressure, causes anemia and vascular stiffness and calcification, and so on.[16,17] In the present study, the average number of stenotic coronary arteries was significantly bigger in the CKD group compared with the other 2 groups, which may explain at least in part the poor prognosis of CKD patients. CKD patients were older than patients in the other groups, as reported in previous studies,[1,2,16,17] and this may also have affected the severity of CAD because age has been reported to be a risk factor for CAD.[18] In addition, the CKD group had a significantly higher plasma level of BNP, even though blood pressure and LVEF did not differ among the 3 groups. This finding suggests that patients in the CKD group have a larger cardiac overload, although decreased renal clearance of BNP may explain its high plasma level.

On diagnostic CAG, 26.2% of the patients had CKD. In previous general population studies, 17.5% of subjects in the United States, and 10.3% of subjects in Japan were reported to have CKD.[1,19] Compared with those reports, the percentage of patients with CKD found in the present study was very high and may be because they already had a substantial number of coronary risk factors. Furthermore, patients in the CKD group were older than those in the other 2 groups.

During follow-up, the eGFR rate of decline was approximately $3\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}\cdot year^{-1}$. Although eGFR is a function of age, 3 years is too short a period to explain this deterioration. eGFR decreases by only $0.1\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}$ from $60\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}$ if serum Cr in a patient aged 60 years is 1.0 mg/dl. It has been reported that the eGFR rate of decline is approximately $1\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}\cdot year^{-1}$ in Western countries,[20] or $0.36\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}\cdot year^{-1}$ in the Japanese general population.[21] Compared with previous data, the patient groups we analyzed had a very poor prognosis regarding renal function. There was no difference between the 2 groups in the usage of ACEI or ARB, which are known to have a renal protective effect. Multivariate analysis showed that the number of stenotic coronary arteries was significantly associated with the percent eGFR deterioration, whereas age, the total number of CAG and PCI during follow-up, and LVEF were not. This finding suggests that CAD can independently affect the prognosis of renal function.

It has been suggested that atherosclerosis causes renal dysfunction. Our study confirms that common mechanisms promote CAD and CKD. O'Hare et al showed that the frequency of increased Cr was significantly higher in those with a reduced ankle–brachial blood pressure index among subjects who participated in the Atherosclerosis Risk in Communities (ARIC) Study.[9] Elsayed et al[10] monitored renal function for 9.3 years on average in subjects from the ARIC Study and the Cardiovascular Health Study. In patients with cardiovascular disease, the odds ratio for worsening of renal failure was significantly high. Furthermore, in the Framingham Heart Study the new onset of renal disease was closely related to the coexistence of coronary risk factors.[11] These findings imply that the presence of atherosclerosis is a risk factor for worsening of renal dysfunction.

Prevalence of diabetes mellitus and a high systolic blood pressure also showed a significant association with reduced eGFR. It has been demonstrated that renal dysfunction worsens more rapidly in diabetic patients[22] and hypertensive patients.[23] Moreover, multivariate analysis revealed a significant relation between baseline eGFR and the rate of reduction in eGFR, which means that the reduction in eGFR in 3 years was greater in patients with a greater baseline eGFR. The reason for this cannot be clarified from the data obtained in the present study. However, it is possible that the decrease in eGFR in diabetic patients in the state of glomerular hyperfiltration may be even greater.

Another possible explanation for the worsening of renal dysfunction in patients with severe CAD is that they may be more exposed to contrast medium. Contrast medium-induced acute kidney injury (AKI) is a serious iatrogenic complication after CAG or PCI. In previous studies, contrast medium-induced AKI was reported as an increased risk of death or late cardiovascular events.[24,25] CKD, diabetes mellitus, and larger volumes of contrast medium administered in a single procedure were demonstrated to be independent risk factors for contrast medium-induced AKI.[26] There have been no reports regarding whether procedural times of CAG and PCI affect the long-term prognosis of renal dysfunction. However, in the present study contrast medium did not seem to cause the eGFR deterioration observed in the patients with multivessel CAD because the procedural times of CAG and PCI were not independent determinants. We examined the effect of the cumulative amount of contrast media administered in 3 years and did not find a significant relationship between eGFR deterioration and the amount of contrast media ($r=0.06$, NS, $n=318$). We could not collect information regarding whether any patient developed AKI after the first CAG or not. AKI itself may have an effect on the long-term prognosis of renal function.

Study Limitations

In the original definition by the K/DOQI,[27] CKD is defined

as GFR <60 ml·min^{-1}·1.73 m^{-2} or having markers of kidney damage even without GFR decrease. Proteinuria has been sometimes referred as a marker of kidney damage, and in the previous studies, proteinuria has been reported to be a possible marker predicting prognosis of renal function.[21] In the present study, we did not include proteinuria in the definition of CKD in order to concentrate on the change in GFR of the patients with CAD over the 3-year period, but this may give some weakness to our data.

There is a common tendency to refrain from catheter examinations of patients with decreased GFR because of the possibility of inducing an acute deterioration by the use of contrast media. This might have brought some bias to the present study because patients with a more severe clinical presentation tended to undergo CAG even if they had decreased GFR. However, the eGFR was normally distributed in the present study, as reported for the Japanese general population,[28] so the bias, if present, may be small.

Conclusion

Patients with CKD have more severe CAD, which may be why there is a high rate of cardiovascular events in CKD patients, that is, the so-called cardiorenal association. Moreover, patients with more severe CAD had a poor prognosis for renal function itself. CAD seemed to be an independent risk factor for worsening of renal dysfunction.

Acknowledgment

This study was supported in part by research grants from the Japan Science Technology Agency (Core Research for Evolutionary Science and Technology) to Y.H.

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Final Report on Low-Dose Estramustine Phosphate (EMP) Monotherapy and Very Low-Dose EMP Therapy combined with LH-RH Agonist for Previously Untreated Advanced Prostate Cancer

Abschlussbericht über die Low-Dose Estramustinphosphat (EMP) Monotherapie und die Very Low-Dose EMP Therapie in Kombination mit einem LH-RH Agonisten für zuvor unbehandelte fortgeschrittene Prostatakarzinome

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Key words

- low-dose EMP monotherapy
- very low-dose EMP with LH-RH agonist
- previously untreated prostate cancer
- advanced prostate cancer
- estramustine phosphate

Abstract

Purpose: In order to assess the efficacy and toxicity of oral estramustine phosphate (EMP) administration, low-dose EMP monotherapy (study 1) and very low-dose EMP therapy with luteinizing hormone-releasing hormone (LH-RH) agonist (study 2) were conducted in previously untreated prostate cancer and long-term outcomes were compared between the 2 study groups.

Materials and Methods: Studies 1 and 2 were independently performed beginning in June 1999 and November 2001, respectively. Study 1 was composed of 87 patients including 85 assessable patients. All 108 patients recruited for study 2 were assessable. Low-dose EMP monotherapy (2 capsules/day or 280 mg/day) was used in study 1 and very low-dose EMP (1 capsule/day or 140 mg/day) combined with LH-RH agonist was adopted in study 2.

Results: Overall prostate specific antigen (PSA) response rates in studies 1 and 2 were 92.3% and 94.2%, respectively, and overall toxicity rates were 54.1% and 38.9%, respectively. EMP discontinuation due to side effects was encountered more often in study 1 (45.9%) than in study 2 (27.8%). Among the adverse side effects gastrointestinal toxicity was most prevalent in both studies. One patient died of acute pulmonary embolism in study 1, but no one died in study 2. There were 6 cancer deaths in the gastrointestinal tract in study 1 but only 2 cancer deaths in study 2.

Conclusion: Our data indicate that the overall PSA response rate was comparable between both studies. However, rates in overall toxicity and drug discontinuation were higher in study 1 than in study 2. We consider that study 2 is more promising for the treatment of previously untreated advanced prostate cancer, although the rate of adverse side effects is still high as compared with other hormonal therapies. In order to overcome the high toxicity rate, especially the gastrointestinal toxicity, we recently elaborated a method employing tailor-made medicine using SNPs of 1A1 gene in cytochrome P-450 for decreasing the rate of gastrointestinal toxicity. Using this method of patient selection, study 3 has been successfully launched on September 2005 with high drug compliance. Better clinical results are being accumulated.

Introduction

In the early 1970s, estramustine phosphate (EMP) was introduced as a potent anticancer drug for prostate cancer. It was originally produced to allow selective delivery of the alkylating agent into estrogen receptor-positive cancer cells [1]. The compound was initially considered to have dual estrogenic and cytotoxic activity [2]. Although the cytotxic activity was thought to be due to nornitrogen mustard, EMP was later proved to interfere with cellular microtubule dynamics but devoid of alkylating effect. After oral intake, EMP is immediately dephosphorylated at the C17 position of the steroid to yield estramustine and estromustine in vivo. Estramustine preferentially enters prostate epithelial cells where it binds to cellular components of tubulin as well as to microtubule-associated proteins (MAPS) [1], which are essential to growth of microtubules [3]. In addition, estramustine and estromustine are further metabolized to estradiol and estrone, respectively, which suppress the pituitary-gonadal axis, resulting in decline of plasma testosterone level.

Since EMP introduction, it has been evaluated mainly for hormone-refractory advanced prostate cancer (HRPC) alone or in combination with a variety of anticancer agents [4–8], because EMP exhibits a potent activity against prostate cancer even after treatment failure with conventional hormone therapies. Many investigations have

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DOI 10.1055/s-0029-1224657
Aktuel Urol 2010; 41: S34–S40

ISSN 001-7868

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confirmed approximately a 30%–35% objective response rate in HRPC patients on EMP monotherapy of 560 to 1260 mg/day (4–9 capsules/day) in 2 to 3 divided doses [4]. An EMP-based chemotherapy regimen, especially in combination with docetaxel, exhibited a satisfactory overall PSA response rate of 77% with median survival time of 16.8 months [9], and many investigators are searching for more promising regimens to achieve better outcomes.

On the other hand, a small number of trials have been performed using EMP monotherapy for previously untreated advanced prostate cancer (PUAPC). In 1980, Andersson et al. [10] reported a meta-analysis of 228 PUAPC patients on conventional EMP monotherapy (conventional dosage is generally defined to administer 4–9 EMP capsules/day). They found an overall response rate of as high as 84%. However, adverse side effects were severe and very frequent, especially in gastrointestinal (35%–46%) [11, 12] and cardiovascular (36%) [13] toxicities, some of which were fatal. The high frequency of serious adverse side effects prompted the EORTC group (European Organization for Research and Treatment of Cancer) to attempt low-dose EMP monotherapy (280 mg/day or 2 capsules/day) for PUAPC in 1984 [14] because the toxicity was known to decrease in a dose-dependent manner. They found low-dose EMP monotherapy very effective for PUAPC with overall response rate of 89%, which was comparable to that of DES (diethylstilbestrol) monotherapy of 3 mg/day. However, adverse side effects were marked in both monotherapies of EMP and DES, whose cardiovascular fatalities amounted to 5 out of 125 and 9 out of 123, respectively. The frequent severe adverse side effects discouraged urologists to pursue further EMP monotherapy as well as DES monotherapy. At around the same time, LH-RH agonist was developed and introduced into clinical practice. It showed a high response rate (86%–88%) with virtually no toxicity compared with EMP monotherapy [15, 16]. Thereafter, EMP treatment was virtually waived for treating PUAPC patients even in low-dose monotherapy.

Recently, we re-evaluated the efficacy of EMP monotherapy for PUAPC patients [9] because it was elucidated in 1990 that EMP forms insoluble calcium phosphate salt when taken with dairy products [17], by which mechanism the amount of EMP absorption from the intestine could be reduced. If it is the case, the dose of EMP could be greatly lowered by taking EMP without concomitant intake of meals or dairy products. By using this mode of drug administration, we expected that low-dose EMP could achieve adequate serum levels to exert the same anticancer effect as conventional dosage without causing severe adverse side effects [18]. On the basis of the above-mentioned rationale, a new protocol was conducted to administer 2 capsules/day for PUAPC patients [9]. This protocol was designated low-dose EMP monotherapy (study 1). However, the preliminary data of study 1 suggested that even 2 capsules/day of EMP can occasionally cause severe adverse side effects after follow-up of 2 years despite very good response rate. Considering from the preliminary data, a second project was undertaken independently from study 1, by adopting a treatment program of very low-dose EMP (1 capsule/day) combined with LH-RH agonist (study 2) in a hope of minimizing adverse side effects as well as maximizing antitumor activity. In this report, studies 1 and 2 are compared on the basis of clinical data obtained for about 5 to 8 years of follow-up on EMP treatment, and the superiority is discussed of study 2 as compared with study 1 in view of adverse side effects and overall survival rate.

Study 1. Low-Dose EMP Monotherapy in PUAPC Patients

Patients and Methods

Patient evaluation and eligibility

Eligible patients had newly found advanced prostate cancer and were required to have a histological diagnosis of adenocarcinoma of the prostate, stage C, D1, or D2. Minimum serum PSA to be entered in this treatment project was 10 ng/mL. Clinical stages were evaluated from digital rectal examination (DRE), transrectal ultrasonography, X-rays, bone scintigraphy, and pelvic computed tomography (CT).

All patients were recruited from our department and 22 affiliated hospitals. Prior to enrollment, each patient underwent a baseline physical examination, including assessment of performance status (PS) according to ECOG Performance Status Criteria. Laboratory data (blood urea nitrogen, creatinine, lactate dehydrogenase, alkaline phosphatase, serum glutamic oxaloacetic transaminase [GOT], glutamic pyruvic transaminase [GPT], testosterone [TST], estradiol [E_2], luteinizing hormone [LH], follicle-stimulating hormone [FSH] and prostate specific antigen [PSA]) were also determined before treatment. After enrollment, the same laboratory tests were performed every week during the first month and once a month thereafter. Radiological examinations were done every 6 months after initiation of the treatment. Eligible patients were required to be within normal CBC and liver function. Patients had to have PS of 0 to 2. Patients could not have significant active concurrent medical illness or malignancy precluding EMP treatment. Particularly, patients who had a history of cardiovascular event or ulcerative disorders in the intestine were excluded from this study. Histamine H_2-receptor antagonist (famotidine 40 mg/day) and aspirin (100 mg/day) were administered concomitantly with EMP for prophylaxis of gastric ulcer and thromboembolism, respectively. All patients were informed of the investigational nature of this study and had to sign and give written informed consent.

Response assessment

To be assessable cases for PSA response, EMP administration was required for more than 8 weeks. When PSA decreased below the detectable level it was designated PSA complete response (CR) and PSA partial response (PR) was defined as normalization of PSA ($0 < PSA < 4$ ng/mL). These responses were confirmed if a single determination of PSA entered the each corresponding level. PSA incomplete response (IR) was indicated as PSA 4 or greater than 4 ng/mL over the entire treatment period. The state of disease progression was defined as either of the followings: regrowth of the prostate, the appearance of new lesions on computed tomography or bone scintigraphy, or a continuous rise in the PSA level of >4 ng/mL on 3 consecutive measurements, where the first determination of PSA 4 or greater than 4 ng/mL was considered PSA failure. In cases of IR, the next point after the nadir was defined as the point of disease progression, where PSA rise in 3 consecutive measurements was detected.

Treatment plan

Patients received oral EMP of 280 mg/day (2 capsules/day) in 2 divided doses. EMP was taken at least 1 hour before or 2 hours after meal or dairy products. Toxicity was graded according to the National Cancer Institute common toxicity criteria. Patients were treated until disease progression, the development of treatment-limiting toxicity or withdrawal of consent. Treatment was discontinued in the presence of grade 3 to 4 adverse side effects.

In these cases, other hormonal therapy such as maximum androgen blockade was started with cessation of EMP. However, in most cases patients were treated at the physician's discretion. All patients are being followed until death.

Statistical analyses

Survival curves were fitted using the Kaplan-Meier method and compared by the Log-Rank test [19]. In two-tailed tests, p-values <0.05 were considered statistically significant. Values were expressed as mean ± standard deviation or as median.

Results

Patient characteristics

Between June 1, 1999 and October 31, 2001, 87 patients were enrolled in study 1. The pretreatment characteristics of these patients are listed in ○ **Table 1**. Of the 87 patients, 85 were assessable for toxicity and 78 were assessable for PSA response, survival, and disease progression. Two patients were lost to follow-up early in this study. In 7 patients, severe toxicity developed within 8 weeks of therapy and they were evaluable only for toxicity. The remaining 78 patients continued to take EMP for at least 8 weeks. In the 85 patients assessable, the median age was 75 years (range 53–89). Patients in clinical stages C, D1 and D2 were 32, 10, and 43, respectively. Adenocarcinoma was well differentiated in 16 patients, moderately in 37, and poorly in 32. Of the 85 assessable patients, 77 had PS of 0 or 1 with an exceptional PS 2 in 8 patients. The median and mean baseline PSA values were 77.1 and 285 ng/mL (range 10–3 910), respectively.

Overall changes in the mean serum PSA, E2, TST, LH, FSH, GOT and GPT

These data had been shown in the earlier reports [19]. Briefly, low dose EMP can quickly and adequately suppress the pituitary gonadal axis and the effect can be maintained for more than 30 months.

Clinical outcomes

As of August 31, 2007, the mean observation time was 56 ± 28 months (range: 1–96) (○ **Table 2**). The mean EMP administration time was 28 ± 23 months (range: 1–96). Of the 78 assessable patients for PSA response, 41 and 31 had complete and partial PSA response, respectively. Total PSA response rate of complete and partial response was 92.3% (72/78), while PSA incomplete response rate was only 7.7% (6/78). During the follow-up period, disease progression was experienced in 29 patients (37.2%), of whom PSA failure was observed in 27 (34.6%), regrowth of the prostate in 1 (1.3%) and new bony lesion in 1 (1.3%). A total of 56 patients died during the observation period. Twenty nine died of prostate cancer including 1 small cell cancer of the prostate, and another 27 died of diseases other than prostate cancer. During the entire follow-up period including a period after withdrawal of EMP administration, 4 died of embolism, 2 in cerebral, 1 in pulmonary and 1 in coronary artery. Furthermore, as many as 6 patients died of gastrointestinal cancers, 2 in the stomach, 1 in the esophagus, 1 in the liver, 1 in the pancreas and 1 in the colon.

Toxicity

Toxicities are listed in ○ **Table 3**. Gastrointestinal symptoms (nausea, vomiting, anorexia, and stomachache) were the most frequently encountered in 23 of 85 cases, followed by 9 congestive heart failures. The third most frequent toxicity was edema in the lower extremities and liver dysfunction each in 6 cases. In addition, 25 cases demonstrated initial transient rise in transaminases less than 150 IU/mL during the first 3 months of treatment, which did not necessitate cessation of EMP administration. The fifth most frequent toxicities were cerebral infarction in 3 followed by 2 pulmonary infarctions. Treatment-related death was seen in 1 case of sudden death (1.2%: 1/85) due to pulmonary embolism. Overall adverse side effects were documented in 46 of 85 cases (54.1%). As a result, EMP treatment had to be discon-

Table 1 Patient characteristics in studies 1 and 2.

	study 1	study 2
	no.	no.
total patients enrolled	87	108
assessable patients	85	108
patients evaluable for		
biological response	78	103
toxicity	85	108
age (yrs)		
median (mean) ± SD	75 (74) ± 8.6	72 (72) ± 8
range	53–89	55–89
stage		
C	32	53
D1	10	11
D2	43	44 n.s. (study 1 vs. 2)
cell differentiation (Gleason Score)		
well (2–4)	16	13
moderately (5–7)	37	45
poorly (8–10)	32	50 n.s. (study 1 vs. 2)
performance status (ECOG)		
PS 0	65	88
PS 1	12	16
PS 2	8	4
PS 3	0	0
PS 4	0	0 n.s. (study 1 vs. 2)
baseline PSA value (ng/mL)		
median (mean) ± SD	77.1 (285) ± 580	70.0 (505) ± 1444
range	10–3910	10–11 000

Table 2 Clinical outcomes in studies 1 and 2.

	study 1	study 2
mean observation time (month)	56±28	49±15
range (month)	1–96	7–68
mean dosage time (month)	28±23	47±15
range (month)	1–96	7–68
PSA response	no. of patients (%)	no. of patients (%)
	n=78	n=103
complete response (PSA: nondetectable)	41 (52.6%)	65 (63.1%)
partial response (0<PSA<4 ng/mL)	31 (39.7%)	32 (31.1%)
incomplete response (PSA≥4 ng/mL)	6 (7.7%)	6 (5.8%)
disease progression*	29 (37.2%)	20 (19.4%)
PSA failure	27 (34.6%)	19 (18.4%)
prostate regrowth	1 (1.3%)	0 (0%)
new bony lesion	1 (1.3%)	0 (0%)
death from		
prostate cancer**	29 (37.2%)	21 (20.4%)
other causes	27 (34.6%)	13 (12.6%)

* $p<0.05$ compared with study 2
** $p<0.01$ compared with study 2

Table 3 Toxicities and EMP discontinuation in studies 1 and 2 for previously untreated advanced prostate cancer patients.

	study 1 (n=85)		study 2 (n=108)	
	no. of EMP discontinuation (%)		no. of EMP discontinuation (%)	
	total (%)		total (%)	
	(grades 1–4)*	(grades 3 and 4)	(grades 1–4)*	(grades 3 and 4)
gastrointestinal symptoms	23 (27.1%)	19 (22.4%)	14 (13.0%)	9 (8.3%)**
congestive heart failure	9 (10.6%)	7 (8.2%)	4 (3.7%)	4 (3.7%)
peripheral edema	6 (7.1%)	3 (3.5%)	7 (6.5%)	4 (3.7%)
liver dysfunction	6 (7.1%)	4 (4.7%)	15 (13.9%)	8 (7.4%)
[initial rise in GOT/GPT	25 (29.4%)		29 (26.9%)]	
cerebral infarction	3 (3.5%)	3 (3.5%)	2 (1.9%)	2 (1.9%)
pulmonary embolism	2 (2.4%)	2 (2.4%)†	0 (0%)	0 (0%)
others	10 (11.8%)	1 (1.2%)	7 (6.5%)	3 (2.8%)
overall toxicities				
adverse side effects	46/85 (54.1%)		42/108 (38.9%)	
EMP discontinuation	82/85 (96.5%)		74/108 (68.5%)	
due to side effects	39/85 (45.9%)		30/108 (27.8%)	
EMP refractory	29/85 (34.1%)		20/108 (18.5%)	
due to other causes	14/85 (16.5%)		24/108 (22.2%)	
drug continuation	3/85 (3.5%)		34/108 (31.5%)	

* National Cancer Institute common toxicity criteria
** One of the 9 patients suffered from severe hematemesis.
† One of the 2 patients died of pulmonary embolism.

tinued in 39 of 85 cases (45.9%) due to severe side effects. Gynecomastia and impotence, though the grade differed from case to case, were experienced in the majority of cases studied.

Study 2. Very Low-Dose EMP Therapy Combined with LH-RH Agonist in PUAPC Patients

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Patients and Methods

Patient evaluation and eligibility as well as response assessment were quite same as study 1.

Treatment plan

Patients took oral EMP dose of 140 mg/day (1 capsule/day) in the morning 2 hours following breakfast without dairy products. After 4 weeks of oral EMP treatment, LH-RH agonist injection (3.75 mg leuprorelin acetate or 3.6 mg goserelin acetate) was initiated and was continued once in every 4 weeks thereafter along with maintenance of oral EMP administration. Other treatment plan was the same as in study 1.

Statistical analyses

Identical to study 1.

Results

Patient characteristics

Between November 1, 2001 and June 30, 2003, 108 patients were enrolled in this treatment project. The pretreatment characteristics of these patients are listed in ○ **Table 1**. Of the 108 patients, all were assessable for toxicity and 103 were assessable for PSA response, survival and disease progression. Five patients suffered from severe toxicity within 8 weeks of therapy and they were

evaluable only for toxicity. The remaining 103 patients continued to take EMP for at least 8 weeks. In the 108 patients assessable, the median age was 72 years (range 55–89). Patients in clinical stages C, D1, and D2 were 53, 11 and 44, respectively. Adenocarcinoma was well differentiated in 13 patients, moderately in 45, and poorly in 50. Of the 108 assessable patients for toxicity, 104 were in PS of 0 or 1 with exceptional PS2 in 4 patients. The median and mean baseline PSA values were 70.0 and 505 ng/mL (range 10–11 000), respectively.

Overall changes in the mean serum PSA, E2, TST, LH, FSH, GOT and GPT

These data had been shown in the earlier reports [19]. Briefly, suppression of the serum PSA was slightly inadequate during the first month of treatment when it was done only with 1 capsule/day of EMP although serum testosterone level decreased to almost castrated level. At the end of first month, LH-RH agonist was injected. In 2 months, the PSA level rapidly declined to very low level along with castrated level of serum testosterone.

Clinical outcomes

As of August 31, 2007, the mean observation time was 49 ±15 months (range: 7–68) (○ **Table 2**). The mean EMP administration time was 47 ± 15 months (range: 7–68). Of the 103 assessable patients for PSA response, 65 and 32 had complete and partial PSA response, respectively. Total PSA response rate of complete and partial response was 94.2% (97/103), while the PSA incomplete response rate was only 5.8% (6/103). During the follow-up period, disease progression was experienced in 20 patients (19.4%), of whom PSA failure was observed in 19 (18.4%) and regrowth of the prostate in 1 (1.0%), but no new bony lesion was noted. A total of 34 patients died during the observation period. Twenty one died of prostate cancer, and another 13 died of diseases other than prostate cancer in which there was no death from vascular embolism. During the entire follow-up period including a period after withdrawal from EMP administration, 4 died of cancer other than the prostate, 2 in the lung, 1 in the liver and 1 in the pancreas. Overall survival (○ **Fig. 1**) in study 2 was significantly higher ($p < 0.01$) than that in study 1, although cause specific survival (○ **Fig. 2**) in study 2 was not significant as compared with that in study 1.

Toxicity

Toxicities are listed in ○ **Table 3**. Liver dysfunction was encountered in 15 out of 108 cases. In addition, there were 29 cases of initial transient rise in transaminases less than 150 IU/mL during the first 3 months which did not necessitated cessation of EMP administration. The second most frequent toxicity was gastrointestinal symptoms in 14 out of 108 cases (nausea, vomiting, anorexia, and stomachache), including 1 case of hematemesis just 7 days after initiation of EMP administration. Peripheral edema was the third most frequent toxicity, which was observed in 7 patients including 1 case of decline in platelet count. The fourth most frequent toxicity was congestive heart failure in 4 cases. However, no cardiovascular embolism was observed except for 2 cases of non-fatal cerebral infarction which was recognized long after cessation of EMP administration. No treatment-related deaths were noted during the entire follow-up period. Overall adverse side effects were recorded in 42 of 108 cases (38.9%), of which 30 cases (27.8%) had to discontinue EMP administration. Slight gynecomastia and high-grade impotence were documented in most cases.

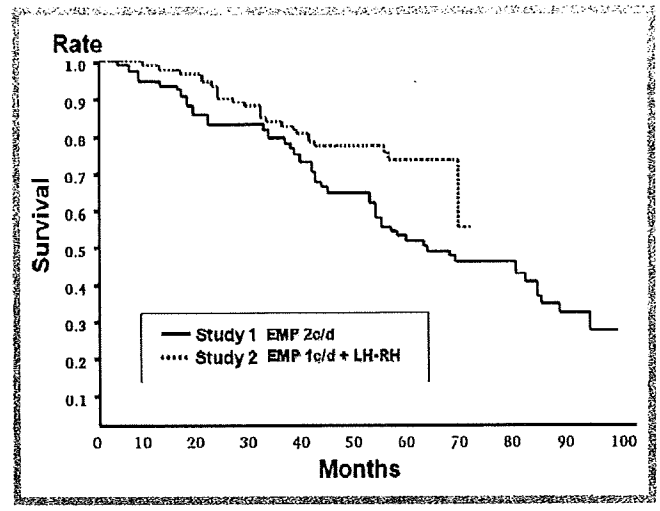

Fig. 1 Overall survival in two studies. Kaplan–Meier plot demonstrates that those patients in study 2 (EMP 1 cap daily added to LH-RH analogue therapy) (dotted line) have longer time to death from time of clinical relapse than those patients in study 1 (EMP 2 cap daily). Log-rank regression analysis shows ; $p < 0.01$.

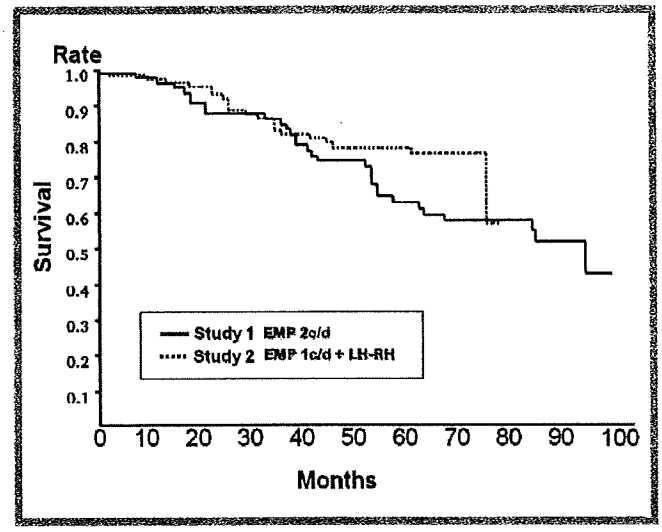

Fig. 2 Cause specific survival in two studies. Kaplan–Meier plot demonstrates that there is no difference between these 2 studies. Log-rank regression analysis shows; $p = 0.19$.

Discussion

At the beginning of this series of EMP therapy, low-dose EMP monotherapy was considered safe and effective based on the assumption that even low-dose EMP will exert an anticancer effect and maintain adequate serum level of EMP by avoiding concomitant intake with dairy products. However, serum estradiol level that is a good indicator of serum EMP level rose to a very high level after 2 capsule/day of EMP intake, with which a considerable number of side effects developed. Resultantly, drug compliance diminished in study 1, in spite that the response rate was satisfactorily high (92.3%). For the next step, very low-dose EMP therapy combined with LH-RH agonist (study 2) was employed in an attempt to lessen side effects as well as to achieve high PSA response rate. This assumption appears to have been almost fulfilled in study 2. As the enrollment characteristics in both study groups are comparable with respect to all major

parameters, comparisons between both studies may be beneficial for various EMP therapies that will be performed hereafter. We believe it will not be valueless to compare studies 1 and 2, in spite that these studies were independently conducted and non-randomized studies.

Comparison between studies 1 and 2

Clinical outcomes

Total PSA response rates of complete and partial response were excellent both in study 1 (92.3%) and study 2 (94.2%), which is in accordance with our former report (93.4%) [9], and is rather superior to other reports (62%-94%) [14, 15, 20–25]. PSA incomplete response (7.7%) in study 1 was as low as that (5.8%) in study 2. During the follow-up period, disease progression was more often encountered in study 1 than in study 2, though the follow-up period was slightly longer in the former than the latter. Overall survival rate is significantly higher in study 2 than in study 1 (○ **Fig. 1**), however, cause specific survival was not significant between the 2 studies (○ **Fig. 2**). The reason of this is not clear but we speculate that the difference will be significant if the follow-up period became longer.

Toxicity

Overall toxicities were more common (54.1%) in study 1 than those (38.9%) in study 2. Takenaka et al. [21] reported that overall toxicity rate was 55% on EMP monotherapy of 4 capsules/day. Taking account of these data into consideration, toxicity of EMP seems to increase in a dose dependent manner like serum estradiol level does. Moreover, 1 case of fatal pulmonary embolism was seen in study 1, while no such serious adverse side effects were encountered in study 2. In the report of EORTC [14], cardiovascular side effects were documented in 36% including 5 fatal cases (4%) on low-dose EMP monotherapy, while there were 12 deaths (9.8%) on DES monotherapy. These data suggest that our studies are superior to EORTC in terms of fatal cardiovascular toxicity, which is more prominent in study 2. Likewise, gastrointestinal toxicity (GIT) in study 1 (27.1%), which is in accordance with the report of EORTC (26%) [14], was much higher than that in study 2 (13.0%). In addition, GIT reported by Takayasu et al. [20] is 36%, which was conducted on 4 capsules/day of EMP. These data support that GIT depends on EMP dosage, too. EMP administration was discontinued due to adverse side effects in 39/85 (45.9%) in study 1 and in 30/108 (27.8%) in study 2. It is likely that side effects requiring EMP discontinuation occur more often in study 1 than in study 2. As a result, toxicities are in general milder in study 2 than in study 1 and depend on EMP dosage. Concerning toxicity, very low-dose EMP administration seems to be superior to other varieties of EMP treatment.

Tailor-made medicine for increasing EMP compliance using gene analysis

There were so many side effects on EMP treatment. GIT was most prominent among all. We inversely used this drawback as a tool for tailor-made medicine to overcome low drug compliance of EMP [26]. Three SNPs, namely m1, m2 and IVS1–728, were selected in cytochrome P-450 1A1 (CYP 1A1) gene to differentiate EMP compliant from non-compliant patients because CYP 1A1 gene has been known to play a role of handling metabolism of estrogen in the liver. In order to identify EMP compliant patients who are supposed to show no GIT on EMP therapy 3 SNPs were evaluated with data obtained from studies 1 and 2. In a result, a crucial relationship was found between 3 SNPs and GIT [26]. If patients carry a major allele (i.e. wild type nucleotide) in each of these 3 SNPs, the risk of GIT is about 12 times higher than patients carrying alleles with other combinations of these 3 nucleotides ($p<0.001$) (○ **Fig. 3**). M2 is only one functional SNP showing non-synonymous amino acid change (valine to isoleucine in codon 462) among 3 SNPs. Currently, we routinely examine those genotypes prior to the EMP therapy in order to exclude patients carrying 3 major allelic combination. Using this method, study 3 has been launched on September 1, 2005 and favorable results are being accumulated.

Summary

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Although both studies 1 and 2 exhibited excellent PSA response, adverse side effects and EMP withdrawal occurred more frequently in study 1 than in study 2. In addition, it was elucidated that not only serum level of estradiol but also the rate of toxicity increased in a dose-dependent manner on EMP therapy. Since most patients found it difficult to take constantly 2 capsules/day of EMP, study 2 is superior to study 1 in every respect. All results of study 2 are comparable or rather superior to those in maximum androgen blockade treatment but those in study 1 are not satisfactory except for PSA response rate. We consider that study 2 is more promising for the treatment of previously untreated advanced prostate cancer, although the rate of adverse side effects is still high as compared with other hormonal therapies. In order to overcome the high toxicity rate, especially the

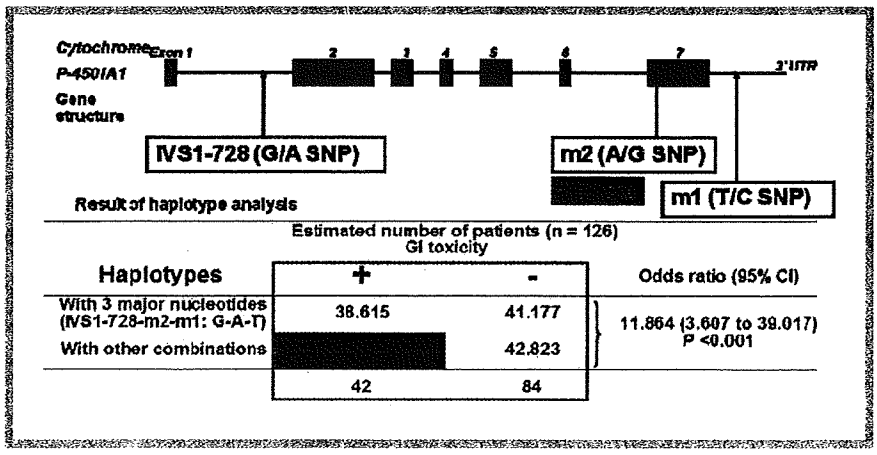

Fig. 3 Single nucleotide polymorphisms (SNPs) in the CYP IA1 gene.
If patients carry an allele with a major nucleotide in each of these 3 SNPs, the risk of gastrointestinal toxicity (GIT) is about 12 times higher than patients carrying alleles with other combinations of these 3 nucleotides ($p<0.001$). Currently, we usually examine those genotypes prior to the EMP therapy in order to exclude patients carrying an allele with these 3 major nucleotides.

GIT, we recently elaborated a method employing tailor-made medicine using SNPs of CYP 1A1 gene for decreasing rate of GIT. Using this method of patient selection, Study 3 has been successfully launched on September 2005 with high drug compliance. Better clinical results are being accumulated.

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