Table 1 Model assumptions

			Base-case value	Range tested in sensitivity analysis (%)	Source
Participant cohort					
Probability (%)					
Falling into sex and age stratum	Male	40–44, 45-49, 50–54, 55–59, 60–64, 65–69, 70–74	10.008, 9.280, 8.810, 9.783, 6.460, 5.721, 4.472	±50	[13]
	Female	40–44, 45-49, 50–54, 55–59, 60–64, 65–69, 70–74	6.291, 6.054, 6.137, 7.364, 6.836, 7.143, 5.643		
Falling into initial renal function stratum	–	Stage 1, stage 2, stage 3, stage 4, stage 5	11.660, 46.095, 28.627, 0.224, 0.029	±50	Japan Tokutei-Kenshin CKD Cohort 2008
	±	Stage 1, stage 2, stage 3, stage 4, stage 5	0.866, 3.771, 3.214, 0.056, 0.008		
	1+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.325, 1.548, 1.779, 0.086, 0.013		
	2+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.080, 0.385, 0.705, 0.095, 0.026		
	≥3+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.027, 0.104, 0.204, 0.053, 0.020		
Decision tree					
Probability (%)					
Seeking detailed examination after screened as further examination required			40.0	±50	[15, 16] and expert opinion
Either eGFR <50 ml/min/1.73 m^2 or having comorbidity among stage 3 patients (advanced stage 3)			83.5	±50	Japan Tokutei-Kenshin CKD Cohort 2008
Starting CKD treatment after detailed examination	–	Advanced stage 3, stage 4, stage 5	48.9, 82.2, 96.0	±50	Delphi method survey of expert committee
	±	Advanced stage 3, stage 4, stage 5	51.7, 83.9, 97.1		
	1+	Stage 1, stage 2, early stage 3, advanced stage 3, stage 4, stage 5	25.6, 31.1, 46.7, 71.7, 92.2, 98.0		
	2+	Stage 1, stage 2, early stage 3, advanced stage 3, stage 4, stage 5	62.2, 68.3, 78.9, 93.2, 97.1, 99.8		
	≥3+	Stage 1, stage 2, early stage 3, advanced stage 3, stage 4, stage 5	93.2, 94.3, 97.1, 97.7, 99.9, 99.9		
Markov model					
Probability (%)					
From (1) screened and/or examined to (2) ESRD with no treatment by initial renal function	–	Stage 1, stage 2, stage 3, stage 4, stage 5	0.001, 0.004, 0.016, 0.154, 1.743	±50	Calculated from Okinawa database [18]
	±	Stage 1, stage 2, stage 3, stage 4, stage 5	0.019, 0.020, 0.036, 1.137, 5.628		
	1+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.036, 0.024, 0.303, 3.527, 15.802		
	2+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.080, 0.305, 1.170, 10.939, 31.409		
	≥3+	Stage 1, stage 2, stage 3, stage 4, stage 5	0.347, 0.933, 2.506, 13.824, 69.340		

Table 1 continued

				Base-case value	Range tested in sensitivity analysis (%)	Source
From (2) ESRD to (5) death by sex and age	Male		40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90	0.033, 0.034, 0.035, 0.036, 0.038, 0.039, 0.041, 0.042, 0.044, 0.045, 0.047, 0.048, 0.050, 0.052, 0.054, 0.056, 0.058, 0.060, 0.062, 0.065, 0.068, 0.071, 0.074, 0.078, 0.081, 0.084, 0.088, 0.092, 0.097, 0.101, 0.105, 0.111, 0.117, 0.123, 0.129, 0.135, 0.142, 0.148, 0.155, 0.160, 0.166, 0.176, 0.186, 0.196, 0.202, 0.208, 0.226, 0.229, 0.245, 0.288, 0.257	±50	Calculated from Japanese dialysis patient registry [21]
	Female		40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90	0.029, 0.030, 0.031, 0.032, 0.033, 0.034, 0.035, 0.036, 0.038, 0.039, 0.041, 0.042, 0.043, 0.045, 0.047, 0.049, 0.050, 0.052, 0.055, 0.057, 0.059, 0.062, 0.065, 0.068, 0.070, 0.074, 0.078, 0.080, 0.085, 0.089, 0.093, 0.097, 0.101, 0.105, 0.110, 0.115, 0.122, 0.127, 0.134, 0.138, 0.145, 0.151, 0.159, 0.162, 0.173, 0.185, 0.188, 0.198, 0.205, 0.219, 0.236		
From (1) screened and/or examined to (3) heart attack with no treatment by initial dipstick test result, sex and age	<1+	Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.005, 0.041, 0.076, 0.132, 0.126, 0.068	±50	[22]
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.019, 0.078, 0.130, 0.234, 0.275, 0.372		
	≥1+	Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.000, 0.000, 0.018, 0.033, 0.112, 0.077		
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.003, 0.010, 0.048, 0.079, 0.211, 0.224		
From (3) heart attack to (5) death by sex and age	1st year	Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	2.8, 13.4, 13.0, 19.5, 33.7, 33.3	±50	[22]
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	33.3, 0.0, 16.9, 25.0, 36.6, 45.8		
	2nd year	Male and female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	3.8, 3.8, 6.7, 19.5, 41.2, 100.0	±50	[24]
From (3) heart attack/(4) stroke to (2) ESRD				0.202	±50	[27]
From (1) screened and/or examined to (4) stroke with no treatment by initial dipstick test result, sex and age	<1+	Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.026, 0.139, 0.264, 0.477, 0.738, 0.769	±50	[22]
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.050, 0.202, 0.357, 0.655, 1.052, 1.540		
		Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.014, 0.083, 0.124, 0.271, 0.508, 0.570		
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	0.034, 0.133, 0.187, 0.382, 0.699, 0.905		
From (4) stroke to (5) death by sex and age	1st year	Male	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	19.1, 14.3, 9.9, 10.6, 12.7, 18.2	±50	[22]
		Female	40–44, 45–54, 55–64, 65–74, 75–84, ≥85	13.6, 14.0, 13.7, 6.8, 14.8, 18.1		
	2nd year	Male	40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, ≥85	6.8, 8.2, 9.5, 12.6, 16.6, 23.3, 37.6, 61.9, 95.1, 100.0	±50	Calculated from Suzuki et al. [25, 26]
		Female	40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, ≥85	5.4, 6.4, 7.5, 9.0, 12.5, 18.4, 26.4, 40.1, 52.6, 71.7		

Table 1 continued

			Base-case value	Range tested in sensitivity analysis (%)	Source
From (1) screened and/or examined to (5) death by sex and age	Male	40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, 85–89, 90–94, 95–99, 100	0.002, 0.003, 0.004, 0.007, 0.010, 0.015, 0.024, 0.042, 0.070, 0.119, 0.196, 0.284, 0.397	±50	[28]
	Female	40–44, 45–49, 50–54, 55–59, 60–64, 65–69, 70–74, 75–79, 80–84, 85–89, 90–94, 95–99, 100	0.001, 0.001, 0.002, 0.003, 0.004, 0.006, 0.010, 0.019, 0.036, 0.070, 0.132, 0.213, 0.327		
Effectiveness of treatment (%)					
Reduction of transition probabilities from (1) screened and/or examined to (2) ESRD with treatment of CKD			42.1	±50	[20]
Reduction of transition probabilities from (1) screened and/or examined to (3) heart attack with treatment of CKD			71.0	±50	[23]
Reduction of transition probabilities from (1) screened and/or examined to (4) stroke with treatment of CKD			69.3	±50	[23]
Quality of life adjustment					
Utility weight					
(1) Screened and/or examined	Stage 1, stage 2, stage 3, stage 4, stage 5		0.940, 0.918, 0.883, 0.839, 0.798	±20	[31]
(2) ESRD			0.658	±20	[32]
(3) Heart attack			0.771		
(4) Stroke			0.714		
Costing					
Annual cost per person (¥)					
Screening	Dipstick test only, serum Cr assay only, dipstick test and serum Cr		267, 138, 342	±50	Survey of health checkup service providers
Detailed examination			25,000	±50	Expert opinion
CKD treatment	Stage 1, stage 2, stage 3, stage 4, stage 5		120,000, 147,000, 337,000, 793,000, 988,000	±50	Expert opinion
ESRD treatment			6,000,000	±50	[33]
Heart attack treatment	1st year, 2nd year		2,780,000, 179,000	±50	[34]
Stroke treatment	1st year, 2nd year		1,000,000, 179,000	±50	[34]

Decision tree

Figure 1a shows our decision tree comparing a do-nothing scenario with a screening scenario. After the decision node, participants under the do-nothing scenario follow the Markov model shown in Fig. 1b. For those under the screening scenario, three types of screening test are considered: (a) dipstick test to check proteinuria only, (b) serum Cr assay only and (c) dipstick test and serum Cr assay. Other tests such as microalbuminuria and cystatin C [14] are not considered, because they are not available options in the context of this study.

Screened participants are portioned between CKD patients who undergo treatment and those who are left untreated through three chance nodes. The first chance node divides the participants between those who require further examination and those left untreated. Participants with (a) dipstick test only, ≥1+; with (b) serum Cr assay only, ≥stage 3; and with (c) dipstick test and serum Cr assay, either ≥1+ or ≥stage 3, are screened as requiring further examination. Those screened as requiring no further examination follow the Markov model. These are implemented by initial renal function stratum.

The second chance node divides participants screened as requiring further examination into those who seek detailed examination at health care providers and those who avoid any further examination. Its probability is assumed at 40.0% based on the literature [15, 16] and of the opinion of an expert committee set up for the purpose of this study, whose members are acknowledged in the "Acknowledgements" section. Those who avoid further examination follow the Markov model.

The third chance node divides participants who underwent further examination into those who undergo treatment of CKD and those left untreated. We derived these probabilities by initial renal function stratum with a Delphi survey of the expert committee. Regarding the strata of stage 3 CKD, a cut-off value of eGFR (50 ml/min/1.73 m^2) and comorbidity such as hypertension, diabetes and/or hyperlipidaemia are considered in order to depict the difference in clinical practice when recommending start of treatment [17]. We label early stage 3 CKD and advanced stage 3 CKD according to this criterion. Among stage 3 CKD patients, the probability of falling into advanced stage 3 CKD by either eGFR <50 ml/min/1.73 m^2 or having comorbidity is 83.5%, calculated from the Japan Tokutei-Kenshin CKD Cohort 2008. Each value is shown in Table 1. All participants follow the Markov model after their completion of detailed examination.

Markov model

The Markov model consists of five health states: (1) screened and/or examined, (2) ESRD, (3) heart attack, (4) stroke and (5) death. Transitions between these states are indicated by arrows. Although individuals follow various courses other than these five health states and indicated transitions, we model in this way based on available data and literature.

We set the span of staying in each state of the Markov model at 1 year. Annual transition probabilities from (1) screened and/or examined to (2) ESRD with no treatment by the initial renal function stratum are calculated from our database of screened cohort in Okinawa Prefecture [18] for this study, since there is no operational predictive model for progression of CKD to ESRD such as Tangri et al. [19] in Japan. Each value is shown in Table 1. Reductions of these transition probabilities brought about by treatment of CKD

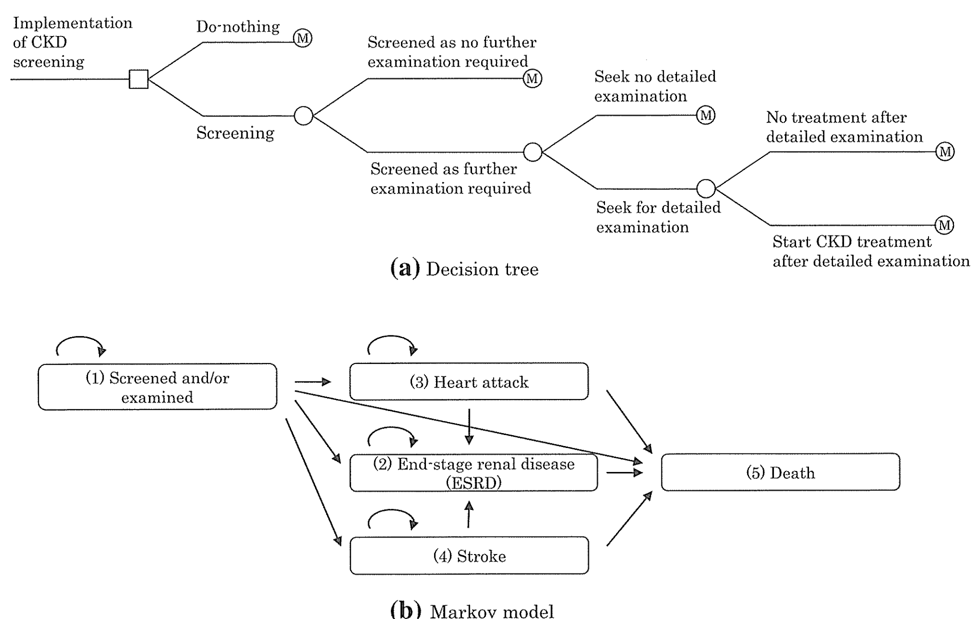

Fig. 1 Economic model. Ⓜ: Markov model

are set at 42.1% based on Omae et al. [20], who investigated the effectiveness of angiotensin-converting enzyme inhibitor in improving renal prognosis. This is a unique Japanese evidence of treatment effectiveness evaluating progression to ESRD which can be compared with our Okinawa cohort [18]. The subsequent transition probabilities to (5) death are calculated from the life expectancy of dialysis starters according to a complete count report of Japanese patients on dialysis [21] by sex and age. Each value is shown in Table 1.

Transition probabilities from (1) screened and/or examined to (3) heart attack with no treatment are adopted from an epidemiological study in Okinawa by Kimura et al. [22] by initial dipstick test result, age and sex. Each value is shown in Table 1. Reductions of these transition probabilities brought about by treatment of CKD are set at 71.0% based on the Hisayama study by Arima et al. [23]. The subsequent transition probabilities to (5) death are adopted from Kimura et al. [22] by age and sex for the first year, and from Fukiyama et al. [24] for the second year and thereafter. Each value is shown in Table 1.

Transition probabilities from (1) screened and/or examined to (4) stroke with no treatment are adopted from Kimura et al. [22] by initial dipstick test result, age and sex. Each value is shown in Table 1. Reductions of these transition probabilities brought about by treatment of CKD are set at 69.3% based on Arima et al. [23]. The subsequent transition probabilities to (5) death are adopted from Kimura et al. [22] by age and sex for the first year, and calculated from the Stroke Register in Akita of Suzuki [25, 26] for the second year and thereafter. Each value is shown in Table 1.

A transition probability from (3) heart attack and (4) stroke to (2) ESRD is adopted from an epidemiological study in Okinawa by Iseki et al. [27].

Transition probabilities from (1) screened and/or examined to (5) death are adopted from Vital Statistics of Japan 2008 [28] by age and sex. Each value is shown in Table 1.

We take a life-long time horizon so that the Markov cycle is repeated until each age stratum reaches 100 years old.

Quality of life adjustment

In order to estimate outcomes, use of quality-adjusted life years (QALYs) is recommended for economic evaluation of health care [29, 30]. QALYs are calculated as the sum of adjusted life-years experienced by a patient, where the adjustment is made by multiplying time by weights linked to the changing health state of the patient. The quality-adjustment weight is a value between 1 (perfect health) and 0 (death), which is one of the health-related quality of life measurements. Regarding (1) screened and/or examined, weights are assigned according to CKD stage based on initial renal function, using values adopted from Tajima et al. [31]. Weights for (2) ESRD, (3) heart attack and (4) stroke are cited from a past economic evaluation of anti-hypertensive treatment in Japanese context by Saito et al. [32].

Costing

From the societal perspective, costing should cover the opportunity cost borne by various economic entities in society. In the context of this study, costs borne by social insurers and patients are considered, since the cost of SHC is borne by social insurers and the cost of treatment is shared by social insurers and patients in Japan's health system. The amount of direct payments to health care providers by these entities is estimated as costs, while costs of sector other than health and productivity losses are left uncounted in this study. Cost items are identified along the decision tree and Markov model: screening, detailed examination, treatment of CKD, treatment of ESRD, treatment of heart attack and treatment of stroke. Each value is shown in Table 1.

Costs of screening were surveyed in five prefectures by inquiring health checkup service providers' price of adding CKD screening test to a test package that does not include renal function tests. Average price of those for (a) dipstick test to check proteinuria only, (b) serum Cr assay only and (c) dipstick test and serum Cr assay was ¥267 (US $3.0, with US $1 = ¥90), ¥138 (US $1.5) and ¥342 (US $3.8) per person, respectively. Cost of detailed examination is set at ¥25,000 (US $278) per person according to the national medical care fee schedule and a treatment model developed by the expert committee. Annual costs of CKD treatment per person are set at ¥120,000 (US $1,333) for stage 1 CKD, ¥147,000 (US $1,633) for stage 2 CKD, ¥337,000 (US $3,744) for stage 3 CKD, ¥793,000 (US $8,811) for stage 4 CKD and ¥988,000 (US $10,978) for stage 5 CKD, also from the national medical care fee schedule and a treatment model developed by the expert committee. Annual cost of ESRD treatment per person, ¥6,000,000 (US $66,667), is cited from a review of renal disease care in Japan by Fukuhara et al. [33]. Annual cost of heart attack treatment per person, ¥2,780,000 (US $30,889) for the first year and ¥179,000 (US $1,989) for subsequent years, are cited from a past economic evaluation of cardiovascular disease prevention in Japanese context by Tsutani et al. [34]. Similarly, annual costs of stroke treatment per person, ¥1,000,000 (US $11,111) for the first year and ¥179,000 (US $1,989) for subsequent years, are cited from Tsutani et al. [34] as well.

Discounting

Both outcomes and costs are discounted at a rate of 3% [30].

Policy options for economic evaluation

To draw significant policy implications from this economic evaluation, policy options from status quo need to be defined. Under the current SHC, the dipstick test to check proteinuria is mandatory, while serum Cr assay is not. However, some health insurers voluntarily provide serum Cr assay to participants in addition to SHC. We surveyed health insurers in five prefectures and found that 65.4% of them implement use of serum Cr assay. Also, we analysed the Japan Tokutei-Kenshin CKD Cohort 2008 and found that 57.3% of participants underwent use of serum Cr assay. Therefore, we define the status quo regarding screening test for CKD as 40% of insurers implementing dipstick test only and 60% implementing dipstick test and serum Cr assay.

Then we evaluate two policy options in this study: 'Policy 1: Requiring serum Cr assay', and 'Policy 2: Requiring serum Cr assay and abandoning dipstick test'. Policy 1 means mandating use of serum Cr assay in addition to the currently used dipstick test, so that 100% of insurers implement both dipstick test and serum Cr assay if policy 1 is taken. Policy 2 is considered based on two recent health policy contexts. One is the discussion aroused during the development of SHC in which requiring serum Cr assay only and abandoning dipstick test used in the former occupational health checkup scheme attracted substantial support. It is expected that such a policy option will be proposed in the revision of SHC. Another relates to the change in diagnosis criterion of diabetes [35], in which a blood test to check the level of haemoglobin A1c instead of a dipstick test to check urinary sugar level has become pivotal. Implementing dipstick test for checking proteinuria only bears scrutiny from the viewpoint of economic evaluation. We assume that 100% of insurers would stop providing dipstick test if policy 2 is adopted.

We calculate incremental cost-effectiveness ratios (ICERs) for these two policy options using our economic model. ICER is a primary endpoint of cost-effectiveness analysis, which is defined as follows:

$$\mathrm{ICER} = \frac{\text{Incremental cost}}{\text{Incremental effectiveness}} = \frac{\mathrm{Cost}_{\text{New policy}} - \mathrm{Cost}_{\text{Status quo}}}{\mathrm{Effectiveness}_{\text{New policy}} - \mathrm{Effectiveness}_{\text{Status quo}}}$$

This means the additional cost required to gain one more QALY under new policy.

Sensitivity analysis

Economic modelling is fundamentally an accumulation of assumptions adopted from diverse sources. Therefore, it is imperative to appraise the stability of the model. We perform one-way sensitivity analyses for our model assumptions. Assumed probabilities about the participant cohort, the decision tree and the Markov model are changed by ±50%. Reductions of transition probabilities brought about by treatment are also changed by ±50%. Utility weights for quality of life adjustments are changed by ±20%. Costs are changed by ±50%. Discount rate is changed from 0% to 5%. We also changed our assumption about status quo that 40% of insurers implement dipstick test only and 60% implement dipstick test and serum Cr assay by ±50% as well.

Results

Model estimators

Table 2 presents the model estimators. Under the do-nothing scenario, no patient is screened, with average cost of renal disease care per person of ¥2,125,490 (US $23,617) during average survival of 16.11639 QALY. When (a) dipstick test to check proteinuria only is applied, 832 patients out of 100,000 participants are screened, with additional cost of ¥7,288 (US $81) per person compared with the do-nothing scenario, for additional survival of 0.00639 QALY (2.332 quality-adjusted life days). When (b) serum Cr assay only is applied, 3,448 patients are screened with additional cost of ¥390,002 (US $4,333) per person compared with the do-nothing scenario, for additional survival of 0.04801 QALY (17.523 quality-adjusted life days). When (c) dipstick test and serum Cr assay are applied, 3,898 patients are screened with additional cost of ¥395,655 (US $4,396) per person compared with the do-nothing scenario, for additional survival of 0.04804 QALY (17.535 quality-adjusted life days).

Model estimators of ICERs were calculated as ¥1,139,399/QALY (US $12,660/QALY) for (a) dipstick test only, ¥8,122,492/QALY (US $90,250/QALY) for (b) serum Cr assay only and ¥8,235,431/QALY (US $91,505/QALY) for (c) dipstick test and serum Cr assay.

Cost-effectiveness

Table 3 presents the results of cost-effectiveness analysis. Regarding the status quo that 40% of insurers implement dipstick test only and 60% implement dipstick test and serum Cr assay, 2,837 patients out of 100,000 participants are screened, with average cost of screening and renal disease care per person of ¥2,365,798 (US $212,922) during average survival of 16.14777 QALY. Taking policy 1 that 40% of insurers currently using dipstick test only start use of serum Cr assay screens more patients (3,898).

Table 2 Model estimators

	No. of patients per 100,000 participants	Cost (¥)	Incremental cost (¥)	Effectiveness (QALY)	Incremental effectiveness (QALY)	Incremental cost-effectiveness ratio (¥/QALY)
Do-nothing	0	2,125,490		16.11639		
(a) Dipstick test only	832	2,132,778	7,288	16.12278	0.00639	1,139,399
(b) Serum Cr assay only	3,448	2,515,492	390,002	16.16440	0.04801	8,122,492
(c) Dipstick test and serum Cr assay	3,898	2,521,145	395,655	16.16443	0.04804	8,235,431

Table 3 Results of cost-effectiveness analysis

	No. of patients per 100,000 participants	Cost (¥)	Incremental cost (¥)	Effectiveness (QALY)	Incremental effectiveness (QALY)	Incremental cost-effectiveness ratio (¥/QALY)
Status quo	2,837	2,365,798		16.14777		
Policy 1: requiring serum Cr assay	3,898	2,521,145	155,347	16.16443	0.01666	9,325,663
Policy 2: requiring serum Cr assay and abandoning dipstick test	3,448	2,515,492	149,694	16.16440	0.01663	9,001,414

It costs more, but it gains more. Its incremental cost is ¥155,347 (US $1,726), and its incremental effectiveness is 0.01666 QALY (6.081 quality-adjusted life days), resulting in ICER of ¥9,325,663/QALY (US $103,618/QALY). Taking policy 2 that 40% of insurers currently using dipstick test only start use of serum Cr assay and abandon dipstick test screens more patients (3,448) compared with the status quo as well. It also costs more, but it gains more. Its incremental cost is ¥149,694 (US $1,663), and its incremental effectiveness is 0.01663 QALY (6.070 quality-adjusted life days), resulting in ICER of ¥9,001,414/QALY (US $100,016/QALY).

Stability of cost-effectiveness

One-way sensitivity analyses produce similar results not only between policy 1 and policy 2 but also among three model estimators of ICER. Therefore, we present a tornado diagram of policy 1 as an example in Fig. 2. Ten variables with large change of ICER are depicted. A threshold to judge cost-effectiveness is also drawn, which is according to World Health Organization's (WHO) recommendation, being three times gross domestic product (GDP) per capita [36]. Its value is ¥11.5 million/QALY (US $128 thousand/QALY) gain in 2009 in Japan.

The effectiveness of CKD treatment to delay progression to ESRD is found to be the most sensitive. Decreasing the effect by 50% increases ICER to ¥16,280,537/QALY (US $180,895/QALY). The effectiveness of CKD treatment to prevent stroke is also found to be the 10th largest change of ICER, but its range is limited.

The cost of treatment for stage 5 CKD is found to be the second most sensitive. Increasing the cost by 50% increases ICER to ¥14,404,335/QALY (US $160,048/QALY). The cost of ESRD treatment is found to be the fifth largest change, and the change is in the opposite direction; decreasing this increases ICER. Another cost item depicted is the cost of treatment for stage 3 CKD, which is found to be the sixth largest change.

The discount rate is found to be the third most sensitive. Discounting at a rate of 5% makes ICER ¥11,373,185/QALY (US $126,369/QALY). Since policy 1 can screen CKD patients without proteinuria by use of serum Cr assay, the prognosis of non-proteinuric stage 5 CKD without treatment is found sensitive as the fourth and the seventh largest change. The eighth largest change depicted relates to the prevalence of CKD in participating population, i.e. stage 2 CKD without proteinuria. The ninth largest change is utility weight for ESRD.

Taking the threshold to judge cost-effectiveness, one-way sensitivity analyses alter the interpretation of the results for only three variables: reductions of transition probabilities from (1) screened and/or examined to (2) ESRD with the treatment of CKD; cost of treatment for stage 5 CKD; and transition probability from (1) screened and/or examined to (2) ESRD with no treatment by initial renal function for stage 5 CKD without proteinuria.

Discussion

We conduct a cost-effectiveness analysis of CKD screening test in SHC. Facing the scheduled revision of mandatory test items, we appraise two possible policy options compared with the status quo that 40% of insurers implement dipstick test to check proteinuria only and 60% implement

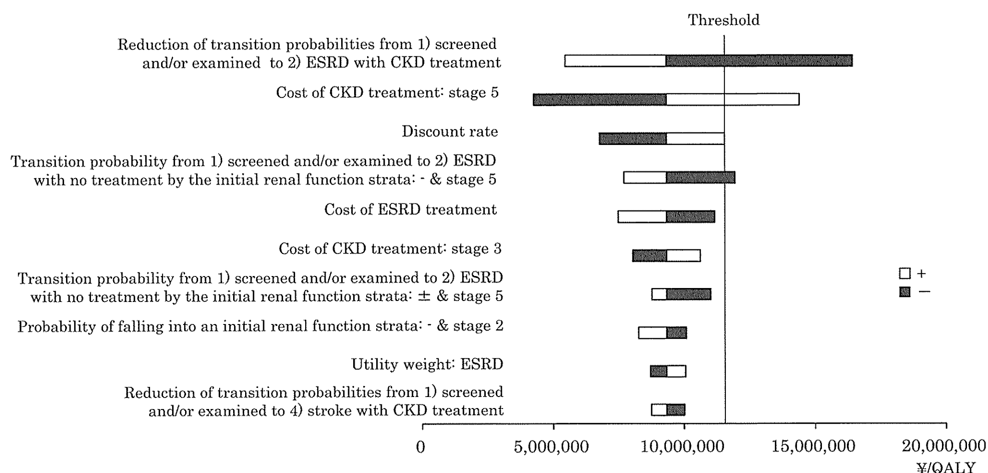

Fig. 2 Tornado diagram of policy 1. This tornado diagram shows ten variables which are found to be sensitive to the change in assumptions. Ten variables are presented, ordered according to the size of the change of ICER from top to bottom. The change of ICERs is represented by *white bars* when increasing the variable or by *black bars* when decreasing the variable from base-case value. The threshold to judge cost-effectiveness is 3 × GDP per capita (¥11.5 million/QALY gain)

dipstick test and serum Cr assay. Policy 1 is to mandate serum Cr assay in addition to the current dipstick test, so that 100% of insurers implement both dipstick test and serum Cr assay. Policy 2 is to mandate serum Cr assay and abandon dipstick test, so that 100% of insurers would stop providing dipstick test and switch to serum Cr assay. Our base-case analysis suggests that both policy options cost more and gain more. Estimated ICERs are ¥9,325,663/QALY (US $103,618/QALY) for policy 1 and ¥9,001,414/QALY (US $100,016/QALY) for policy 2.

To interpret these ICERs, there is no established value of social willingness to pay for one QALY gain in public health programmes such as mass screening in Japan, although some suggest ¥5 million/QALY (US $56 thousand/QALY) for an innovative medical intervention [37]. We follow WHO recommendation in this study, which is three times GDP per capita [36]. Its value is ¥11.5 million/QALY (US $128 thousand/QALY) gain in 2009 in Japan. Given this threshold, both policy 1 and policy 2 are judged as cost-effective. Therefore, mandating serum Cr assay in SHC can be justifiable as an efficient allocation of finite resources for health. Between policy 1 and policy 2, the ICER of policy 2 is slightly more favourable than that of policy 1, while 450 more patients out of 100,000 participants are screened by adopting policy 1. If secondary prevention of CKD is emphasised as a policy objective in addition to efficiency, policy 1 is an acceptable option as well as policy 2.

Our model estimators have a policy implication, although estimated ICERs do not directly depict any marginal change in society. The ICER of (a) dipstick test only compared with the do-nothing scenario, ¥1,139,399/QALY (US $12,660/QALY), is remarkably favourable. This implies that mass screening with dipstick test only is cost-effective compared with abolishment of mass screening for kidney diseases altogether. Therefore, continuing the current policy, i.e. mandatory dipstick test, could be justifiable as an efficient resource allocation.

This contrasts with the reported cost-ineffectiveness of annual mass screening for adults using dipstick test to check proteinuria in the USA [12], although direct comparison cannot be made between the results of economic evaluations under different health systems. The difference could be attributable to the difference in the prevalence of proteinuria among screened population, with 5.450% being used in our model based on the Japan Tokutei-Kenshin CKD Cohort 2008, while 0.19% is assumed in the US study. Such epidemiological differences are known in terms of not only quantity but also in quality [7]. The prevalence of glomerulonephritis, especially IgA nephropathy, is higher in Asian countries including Japan compared with Western countries [10]. Also, the prevalence of renovascular disease such as ischaemic nephropathy, with which patients are often non-proteinuric until advanced stages of CKD, is lower in Asian countries [38]. The inclusion of heart attack and stroke into our model, which are excluded in the US model [12], may have also made the ICER more favourable.

There is a report of cost-ineffectiveness of population-based screening for CKD with serum Cr assay from Canada [39]. This Canadian model can be compared with our model estimators of (b) serum Cr only compared with the do-nothing scenario. Their health outcomes gain or incremental effectiveness is 0.0044 QALY, which is smaller than ours, 0.04801 QALY, while their incremental cost is C $463 (US $441, using US $1 = C $1.05), which is also smaller than ours, ¥390,002 (US $4,333). These differences probably reflect the difference in the prevalence of CKD between Canada and Japan. Regarding the efficiency of screening programme, our model estimator of ICER, ¥8,122,492/QALY (US $90,250/QALY), is slightly more favourable than that of Canada, C $104,900/QALY (US $99,905/QALY). However, the contradictory conclusion regarding cost-effectiveness is not due to this difference but rather the threshold taken. The Canadian study adopts lower value such

as C $20,000 to C $50,000/QALY (US $19,048 to US $47,619/QALY) following local practice [40].

Our sensitivity analysis suggests instability of the results in only three variables, so our findings are robust to a certain extent. The most sensitive variable is the effectiveness of CKD treatment delaying progression to ESRD: 42.1% reduction is adopted in our economic model according to the unique clinical evidence from Japan, whose agent is angiotensin-converting enzyme inhibitor. It is marginally larger than comparative values reported from Western countries. Reductions in the rate of GFR decline are 35.9% by Agodoa et al. [41], 39.8% by The GISEN Group [42] and 22.5% by Ruggenenti et al. [43]. However, we think our assumption of base-case value is reasonable in two accounts: in light of the indication of angiotensin receptor blockers [17], whose use is more tolerated than angiotensin-converting enzyme inhibitors [44], and the higher prevalence of glomerulonephritis including IgA nephropathy, being a primary renal disease for ESRD, in Japan [10], for which the effect of early treatment such as renin–angiotensin system (RAS) inhibition, an immunosuppression, reduces risk of ESRD by 60% [45].

In regards to the other sensitive variables, we think the prognosis of non-proteinuric stage 5 CKD without treatment does not greatly undermine our findings of base-case analysis, since the value is calculated from extended follow-up of an established database [18]. Uncertainty of the base-case value should be much less than the analysed ±50%. On the other hand, the cost of treatment for stage 5 CKD relates to one of the weaknesses of this study, as discussed in the following.

There are weaknesses in this study. The most significant one is that our economic model depicts the prognosis of CKD by initial renal function stratum. This approach is taken because of the limitation of epidemiological data, and it has little difficulty in estimating outcomes in terms of survival. However, it becomes problematic when it comes to costing. For example, a patient initially screened as stage 1 CKD stays at (1) screened and/or examined before transiting to the following health states such as (2) ESRD. This means that a patient skips over stage 2 CKD to 5 CKD before progressing to ESRD. To estimate the cost for this health state, the diversity of patients in terms of progression of the CKD stages should be taken into account. Our expert committee has developed treatment models to understand this problem. This type of uncertainty is larger in stage 1 CKD and smaller in stage 5 CKD, but the cost of stages 1–4 CKD are not found to be so sensitive in our sensitivity analysis. Also, we think that uncertainty of the cost of stage 5 CKD, the second most sensitive variable, is less than the analysed ±50%, and our findings based on the base-case analysis are plausible. The problem also affects quality of life adjustment, which tends to produce larger QALY outcomes.

Table 4 Recommendation of the Japanese Society of Nephrology Task Force for the validation of urine examination as a universal screening

Mandate use of serum Cr assay in addition to the current dipstick test in the next revision of SHC

Other weaknesses include our assumption of 100% adherence to treatment and so on. However, the most significant strength of this study is that our economic model depends totally on evidence from Japan only, which could justify our simplification in modelling on data availability basis. There is an opportunity for further refinement of our economic model, because a large-scale field trial evaluating the effect of multifactorial treatment including lifestyle modification for early-stage CKD [46] is ongoing in Japan, which will enable us to model progression of CKD with more rigorous clinical evidence [47].

In conclusion, we, the Japanese Society of Nephrology Task Force for the Validation of Urine Examination as a Universal Screening, recommend to mandate use of serum Cr assay in addition to the current dipstick test in the next revision of SHC, from the viewpoint of value for money and the importance of secondary prevention (Table 4). We think that continuation of current policy, in which dipstick test only is mandatory, is still a sensible policy option. Development of adequate Specific Counselling Guidance for screened participants is also recommended.

Whereas the primary objective of this study is to appraise policy options in Japanese context, it also demonstrates that good value for money can be expected from mass screening with dipstick test to check proteinuria in population with high prevalence; that is, a population strategy could be adopted for control of CKD. However, caution is needed when extrapolating this conclusion, since the scope of costing of our economic model does not cover the initial cost of launching mass screening. The model here is based on currently running SHC. The practice of annual mass screening for adults in Japan is quite exceptional, while such universal programmes are rarely found in other countries [48].

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Conflict of interest The authors have declared that no conflicts of interest exist.

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ORIGINAL RESEARCH REPORTS

Novel Culture System of Mesenchymal Stromal Cells from Human Subcutaneous Adipose Tissue

Shigejiro Iwashima,[1] Takenori Ozaki,[1] Shoichi Maruyama,[1] Yousuke Saka,[1] Masato Kobori,[4] Kaoru Omae,[5] Hirotake Yamaguchi,[5] Tomoaki Niimi,[6] Kazuhiro Toriyama,[2] Yuzuru Kamei,[2] Shuhei Torii,[2] Toyoaki Murohara,[3] Yukio Yuzawa,[1] Yasuo Kitagawa,[5,6] and Seiichi Matsuo[1]

Accumulating evidence suggests that the delivery of human adipose tissue–derived stromal cells (hASCs) has great potential as regenerative therapy. This was performed to develop a method for expanding hASCs by reducing the amount of serum required. We demonstrate that hASCs were able to expand efficiently in media containing 2% serum and fibroblast growth factor-2. These cells, or low serum cultured hASCs (hLASCs), expressed cell surface markers similar to those on bone marrow–derived mesenchymal stem cells, and could be differentiated into cells of mesenchymal lineage. Of interest, hLASCs secreted higher levels of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) than hASCs cultured in 20% serum (hHASCs). Moreover, hLASC-conditioned media significantly increased endothelial cell (EC) proliferation and decreased EC apoptosis compared to that obtained from hHASCs or control media only. Antibodies against VEGF and HGF virtually negated these effects. When hASCs were administered into the ischemic hindlimbs of nude rats, hLASCs improved blood flow, increased capillary density, and raised the levels of VEGF and HGF in the muscles as compared with hHASCs. In conclusion, we demonstrate a novel low serum culture system for hASCs, which may have great potential in regenerative cell therapy for damaged organs in the clinical setting.

Introduction

Cell therapy has received much attention owing to the ability of stem/progenitor cells to regenerate damaged tissues and organs. To date, many cell types have been presented as promising candidates for regenerative cell therapy, including embryonic stem cells, induced pluripotent stem (iPS) cells [1], and stem/progenitor cells isolated from adult tissues [2]. Although the potential of embryonic stem cells is enormous, many ethical issues accompany their use [3]. Recently established technology to develop iPS cells may overcome these issues. However, much work remains to be done before these cells can be applied to humans.

Concerning adult stem/progenitor cells, many clinical studies have already been performed. In particular, the delivery of bone marrow (BM)-derived stem cells and progenitor cells into damaged tissue to promote regeneration has emerged as a novel therapeutic option [4,5]. Early clinical trials on ischemic tissue suggested that cell therapy using BM-derived cell preparation, containing endothelial progenitor cells (EPCs), could become a viable clinical option [6–10]. However, later studies did not always support these early results [11,12]. The relatively low number of cells obtained in the procedure and the difficulty presented in culturing and expanding EPCs are the main limitations of this approach. In contrast, BM-derived mesenchymal stem/progenitor cells (BM-MSCs) can be expanded ex vivo, and these cells have been shown to differentiate into adipocytes, chondrocytes, osteoblasts, and myoblasts in vitro and in vivo [13,14], making them a promising candidate. In fact, BM-MSCs have been successfully used to promote osteogenesis in patients [15]. Moreover, there is growing evidence from animal studies suggesting that MSCs may have great potential in regenerative cell therapy for ischemic tissue [16]. In spite of all these findings, the clinical use of BM-MSCs has presented many

[1]Department of Nephrology, [2]Department of Plastic Surgery, and [3]Department of Cardiology, Nagoya University Graduate School of Medicine, Showaku, Nagoya, Aichi, Japan.
[4]BioFrontier Laboratories, Kyowa Hakko Kogyo Co. Ltd, Asahi-machi, Machida, Japan.
[5]Innovation Plaza Tokai, Japan Science and Technology Agency, Minami-ku. Nagoya, Aichi, Japan.
[6]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan.

problems, including pain, morbidity, and low cell number upon harvest, which has led many researchers to investigate alternative sources.

Recently, a putative stem cell population has been identified within human adipose tissue [17]. Like BM-MSCs, human adipose tissue–derived stem/stromal cells (hASCs) have the potential to differentiate into various types of cells and tissues [18,19]. Since adipose tissue is abundant, accessible, and replenishable, hASCs have an advantage over BM-MSCs [20] and offer an alternative general source of autologous stem cells for regenerative therapy if the efficiency of stem cell isolation and expansion techniques can be improved. Furthermore, for reasons of safety, reagents of animal origin should be totally eliminated in the process of cell preparation.

The aim of this study was to develop a new method for isolating and expanding hASCs from fat, focusing on a reduction in the amount of serum required. We demonstrated that MSCs can be expanded rapidly and constantly simply by culturing adipose-derived cells in a medium containing 2% serum and fibroblast growth factor-2 (FGF-2). We then characterized these cells and investigated their potential for regenerative medicine (in vitro and in vivo). Here, we report on a novel culture method for hASCs, namely a low serum culture system, which will allow for immediate application to patients.

Materials and Methods

Preparation of culture media

The basal culture medium for hASCs as a 3:2 mixture of Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) and MCDB 201 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 1 mg/mL linoleic acid–albumin (Sigma-Aldrich), one-hundredth volume 100 × ITS supplement (Sigma-Aldrich), 0.1 mM ascorbic acid phosphate ester magnesium salt (Wako Pure Chemical Industries, Osaka, Japan), 50 U/mL penicillin and 50 mg/mL streptomycin (Meiji Seika Ltd., Tokyo, Japan). To this basal medium, fetal bovine serum (FBS) (ICN Biomedicals, Inc, Aurora, OH, USA) was added at concentrations of 0, 1, 2, 5%. Approximately 10, or 20% (vol/vol), and 10 ng/mL human FGF-2 (Peprotech, Inc., Rocky Hill, NJ, USA) epidermal growth factor (EGF, Sigma-Aldrich), insulin-like growth factor-1 (IGF-1, Sigma-Aldrich), or platelet-derived growth factor (PDGF, Sigma-Aldrich) was further added.

Expansion of hASCs from adipose tissue

One to ten grams of abdominal subcutaneous adipose tissue was obtained with written informed consent from 10 patients (six males and four females) undergoing surgery. The average of the patients' age was 62 ± 10, and the BMI was 24.4 ± 2.7 (kg/m^2). All tissue samples were used with approval and according to the guidelines of the ethical committee at the Nagoya University Medical School (Approval number 505–2) and the study was performed according to the guidelines of the Declaration of Helsinki. The specimens were washed with DMEM/F12 medium supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin, and cut into 2-mm cubic pieces. Adipose tissue was digested in 2-mL Hank's balanced salt solution containing 1 mg/mL collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA) with reciprocal shaking for 1 h at 37°C. After removing undigested tissues by passing the samples through nylon mesh with a pore size of 100 mm, the stromal vascular fraction (SVF) was precipitated by centrifugation of the filtrate at 1,200 rpm for 5 min at room temperature. Stromal vascular fraction was washed three times by further centrifugation and suspension in DMEM/F12 medium and the number of nucleated cells was counted by staining SVF with Turk's solution (Nacarai tesque, Inc., Kyoto, Japan). Stromal vascular fraction cells corresponding to 2×10^5 were seeded in 25 cm^2 T-flasks (NUNC) coated with human fibronectin (Sigma-Aldrich) and cultured in 5 mL of media at 37°C under humidified 5% CO_2/95% air. Twenty-four hours later, non-attaching cells, together with the medium, were removed and the culture was continued by feeding fresh medium every other day. All the cells used in this study were negative for mycoplasma (MycoAlert Mycoplasma Detection Kit, Lonza, Walkersville, MD).

Growth speed of hASCs

Subcutaneous adipose tissue obtained from five patients was used for this experiment. Three preparations of 2×10^5 SVF cells from each patient were cultured in the following conditions: a high serum culture medium containing 20% FBS and 10 ng/mL human FGF-2, a low serum culture medium containing 2% FBS and 10 ng/mL human FGF-2, and a high (20% FBS) serum culture medium without FGF-2. Growth kinetics was calculated at each time point and shown as average ± SD. Human serum was obtained from three healthy volunteers, separately filtered through sterilization filters (Corning Inc., Corning, NY), and heat-inactivated (56°C for 30 min). No endotoxins were detected in the sera (Limulus amebocyte lysate chromogenic bioassay; Endospacy Assay, Seikagakukogyo Co., Tokyo, Japan).

Flow cytometry of hLASCs

When the cultures became confluent, they were detached by 0.25% trypsin/EDTA treatment. Aliquots of cell suspension containing 1×10^5 cells were incubated with antibodies dissolved in PBS containing 3% FBS 0.05% NaN_3 for 30 min on ice. Antibodies against the following cell surface markers were used; phycoerythrin (PE)-conjugated Ab against CD13, CD90, CD73, or CD31, and fluorescein isothiocyanate (FITC)-conjugated Ab against CD10, CD44, CD29, CD14, CD45, or Stro1, and Allophycocyanin (APC)-conjugated Ab against CD105, CD34, CD117, or CD133, and periclinin chlorophyll protein cyanine 5 (PerCP-Cy5.5)-conjugated Ab against HLA-DR. All antibodies purchased from Becton Dickson (San Jose, CA, USA). Flow cytometry was performed using fluorescence-activated cell sorting (FACS) Vantage SE (BD Japan, Tokyo, Japan). All cells were stained with nonspecific IgG to assess background fluorescence.

Evaluation of cell morphology

Cell areas of hASCs (no FGF-2), hHASCs, and hLASCs obtained from five patients were measured by tracing cells with the use of MetaMorph 6.3 (Universal Imaging Co., West Chester, PA). The results were shown as a ratio of the average cell area of hHASCs to that of hLASCs obtained from each patient.

Differentiation of hLASCs

Human LASCs at passages 4 through 6 were used for the differentiation study as follows.

Adipogenesis. Adipogenic differentiation was induced as previously described, with minor modification [21].

Cells were incubated with medium containing Dulbecco's modified Eagle's medium (DMEM), 10% (vol/vol) FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 0.1 mM indomethacin (Wako Pure Chemical Industries Ltd.), 1 mM hydrocortisone (Sigma-Aldrich), 10 mg/mL insulin (Sigma-Aldrich), and 1 mM dexamethasone (Sigma-Aldrich) for 2 weeks. Adipogenic differentiation was confirmed using the fluorescent neutral lipid dye BODIPY 493/503 (Molecular Probes, Inc., Eugene, OR).

Osteogenesis. Osteogenic differentiation was induced as previously described, with minor modification [22]. Cells were incubated with medium containing DMEM, 10% FBS, 50 mM ascorbic acid phosphate ester magnesium salt, 10 mM b-glycerophospahte (Sigma-Aldrich), and 0.1 mM dexamethasone for 2 weeks. Osteogenic differentiation was confirmed by assaying von Kossa staining.

Chondrogenesis. Chondrogenic differentiation was induced as previously described, with minor modification [23].

Cells were incubated with medium containing the serum-free DMEM and 100 ng/mL transforming growth factor (TGF)-β. The cells were stained with 1% (wt/vol) Alcian Blue solution (Sigma-Aldrich).

Determination of cytokines secreted from hLASCs or hHASCs

Human LASCs and hHASCs were cultured from five patients, and cells at passages 4 through 6 were used for the experiment. At 90% confluence, the medium was replaced with fresh DMEM containing 10% FBS, and cells were incubated for 24 h. At the end of the incubation period, the conditioned media from hLASCs or hHASCs were collected, and cell numbers were counted using a hemacytometer. The collected media were analyzed for the secretion of vascular endothelial growth factor-A (VEGF-A), hepatocyte growth factor (HGF), and stromal cell–derived factor 1α (SDF-1α), using commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits; Quantikine Human VEGF Immunoassay (R&D Systems, Minneapolis, MN), HGF Otsuka ELISA kit (Otsuka Pharmaceutical Co., Tokyo, Japan), and Quantikine Human SDF-1 α Immunoassay (R&D Systems). Data are expressed as picograms of the secreted factor per 10^6 cells at the time of harvest.

Evaluation of bioactivity of hASC-conditioned media

The effect of the conditioned media from hHASCs or hLASCs on endothelial cell (EC) proliferation was evaluated by MTS assay as previously described [24]. Briefly, hLASCs and hHASCs were cultured and expanded on tissue culture plates in low-serum (2%) or high-serum (20%) condition and used for the experiments at passages 4 through 6. At 90% confluence, the medium was replaced with endothelial basal medium 2 (EBM2, Lonza), containing 0.5% FBS, and cells were incubated for 24 h. 50% conditioned medium (100 μL of 24-h conditioned medium mixed with 100 μL fresh EBM containing 0.5% FBS) was added to human umbilical vein endothelial cells (HUVECs, Takara, Ootsu, Japan) on 96-well plates (1,500 cells/well). The plates were incubated for 48 h at 37°C, and 20 μL of CellTiter 96 Solution Agent (Promega Corporation, Madison, WI, USA) was added to each well. After incubation for 2 h at 37°C, the absorbance at 490 nm was measured using a 96-well plate reader; Spectra 2 (TECAN, Männedorf, Switzerland).

The effect of hASC-conditioned medium on EC apoptosis was evaluated by TUNEL staining as described [25,26]. Briefly, HUVECs were grown in 4-well chamber slides (Nalge Nunc International, Rochester, NY) and incubated for 48 h in hypoxic condition (1% O_2) in the presence or absence of 50% conditioned medium obtained from ASC. The cells were then fixed with 4% paraformaldehyde in PBS, and apoptotic cell death was determined by TUNEL staining using in situ Apoptosis Detection Kit (Takara). Six non-overlapping fields (×200) on the cell culture chamber slides were photographed. The numbers of TUNEL positive cells were counted using MetaMorph 6.3 (Universal Imaging Co., West Chester, PA).

Effects of neutralizing antiobodies against VEGF and HGF on EC

The effects of VEGF and HGF activity in the hLASC- or hHASC-conditioned media were examined by means of VEGF-neutralizing goat polyclonal IgG (R&D Systems) and HGF-neutralizing goat polyclonal IgG (R&D Systems). As for these antibodies, the IgG fraction at a concentration of 10 μg/mL was able to neutralize a biological activity of 10 ng/mL VEGF or 100 ng/mL HGF. Normal goat IgG fraction (10 μg/mL) was employed as a control. These neutralizing antibodies or control were added to conditioned media, and MTS assay or TUNEL assay was performed.

Rat hindlimb ischemia model

Unilateral hindlimb ischemia was created in 15-week-old Male F344/NJcl-*rnu/rnu* rats (CLER Japan Inc., Tokyo, Japan) as described previously [27]. Briefly, rats were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and the unilateral femoral artery was dissected free along its entire length. All branches were ligated and excised. Then, hLASCs or hHASCs (1×10^7/body), or vehicle were carefully injected directly into the ischemic limb of rats right after surgery. All animal protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University.

Laser Doppler blood flowmetry

The ratio of ischemic /normal hindlimb blood flow was measured using laser Doppler blood flowmetry (moorLDI, Moor Instrument, Devon, UK) as described previously [28]. Low to no flow was displayed as dark blue, whereas high blood flow was displayed as red to white. At three predetermined time points (immediately after surgery, and on postoperative days 7 and 14), we performed 2 consecutive laser scannings over the same region of interest (legs and feet). The average flow of the ischemic and nonischemic legs was calculated on the basis of histograms of the colored pixels. To minimize variations due to ambient light, blood flow was expressed as the ischemic (left)/normal (right) limb flow ratio.

Immunohistochemistry

At days 7 and 14, rats were euthanized and thigh muscles were removed from ischemic limbs for histological analysis. Tissue preparation and immunostaining were performed as described [29,30]. Frozen sections were incubated with monoclonal antibodies against rat CD31 (BD Japan, Tokyo, Japan), human CD31 (DakoCytomation, Glostrop, Denmark), human Lamin A/C (Novocastra, Newcastle, UK), and human smooth muscle actin (DakoCytomation), followed by fluorescence-conjugated goat anti-mouse IgG (Sigma-Aldrich).

Measurement of capillary density

Muscles removed at day 14 were stained for rat CD31 and the number of vessels was counted under a light microscope (magnification, 200× in a blinded manner. To ensure that the capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined [28].

Measurement of VEGF, HGF, and SDF-1α concentrations in hindlimb

Three days after inducing ischemia, the hindlimb muscles were removed and homogenized with a QIAGEN MM300 mixer mill (Qiagen, Hilden, Germany) in assay solution. The concentration of human VEGF, human HGF, and human SDF-1α in the hindlimb were measured using a commercial sandwich ELISA kit. The antibody against human VEGF and HGF reacts with only human VEGF and HGF, and not with rat VEGF and HGF. Data are expressed as pg per g protein.

Statistical analyses

Statistical analysis was performed using a software program, Stat View 5.0 (SAS Institute, Cary, NC, USA). Two-way analysis of variance (ANOVA) was used to determine the significant difference among the three groups. When a statistical difference was indicated by ANOVA, further analysis was performed using Fisher to determine the difference between any pair of groups. A significant difference was defined as a P value of <0.05. All values are provided as mean ± SD.

Results

Development of a low serum culture system

Before finding 2% serum and 10 ng/mL FGF-2 to be the best combination, we tested lower serum concentrations. In serum-free medium or 1% FBS medium, SVF cells did not proliferate when cultured with EGF, IGF-1, PDGF, or FGF-2 alone, or in any of their combinations. In culture media containing 2% FBS, the cell expansion stopped between the second and third passages when cultured with EGF, IGF-1, or PDGF alone, or in any combination. In these culture media, cytoplasm of the cells had enlarged before the proliferation arrest. In contrast, using a medium containing 2% FBS and 10 ng/mL FGF-2, repeated passages were possible for more than 60 days over 15 passages. These low serum cultured cells, or hLASCs had small cytoplasm compared to the nucleus, and this morphology was maintained through the repeated passages. An FGF-2 concentration of 10 ng/mL was used in our standard low serum medium, which we confirmed could be reduced to 1 ng/mL. In media containing 5, 10, or 20 FBS, cells were expanded over several passages without addition of any growth factors, with 20% of FBS giving the fastest growth.

Phenotype of hLASCs

FACS analysis revealed that cell surface markers of CD13, CD29, CD44, CD73, CD90, and CD105 were positive on hLASCs, while CD14, CD31, CD34, CD45, CD117, CD133, Stro1, and HLA-DR were negative (Fig. 1A). All of these are consistent with the pattern of surface markers on BM-MSCs as reported previously [31]. Mesenchymal stem cell phenotype of hLASCs was further confirmed by adipogenic, chondrogenic, and osteogenic differentiation (Fig. 1B).

Growth kinetics of hASCs

The growth speeds of hASCs cultured under the three different conditions were assessed. Equal numbers (2×10^5) of SVF cells taken from each patient were placed in culture media containing 20% FBS with or without FGF-2, or in one containing 2% FBS with FGF-2. Human HASCs cultured in the high serum medium (20% FBS with FGF-2) grew the fastest, followed by hLASCs, those cultured in the low serum medium (2% FBS with FGF-2). The growth rate of hASCs (no FGF), which had been cultured in a conventional media (20% FBS without FGF-2) was the lowest (Fig. 2A). These results demonstrate that an autologous hLASCs population of 1×10^8 could be expanded within 21 days of culture starting with 2×10^5 SVF cells obtained from only 1 g of subcutaneous adipose tissues.

We then investigated whether FBS could be replaced by human serum. Stromal vascular fraction cells (2×10^5) were placed in a culture media containing 10 ng/mL FGF-2 and 2% human sera taken from three volunteers. The growth kinetics showed that hASCs also proliferated well in a low serum culture medium using human serum (Fig. 2B).

The morphology of hASCs

Human LASCs were small and round while hHASCs or hASCs (no FGF) were relatively large and flat (Fig. 3A). Cell areas of hASCs cultured under three different conditions were determined using MetaMorph. On average, hHASCs were significantly larger than hLASCs; hASCs (no FGF) were even larger (Fig. 3B).

Cytokine secretion by hLASCs and hHASCs

Since angiogenesis is an important process in the regeneration of any tissue, secretion of angiogenesis-related molecules was analyzed. The levels of VEGF and HGF were significantly higher in the hLASC-conditioned medium than those in the hHASC-conditioned medium. Both hLASCs and hHASCs secreted a significant amount of SDF-1α at similar levels (Fig. 4).

Effects of hASCs on EC proliferation and apoptosis

Because the proliferation and survival of EC is an important aspect of angiogenesis, we studied the effects of ASC-

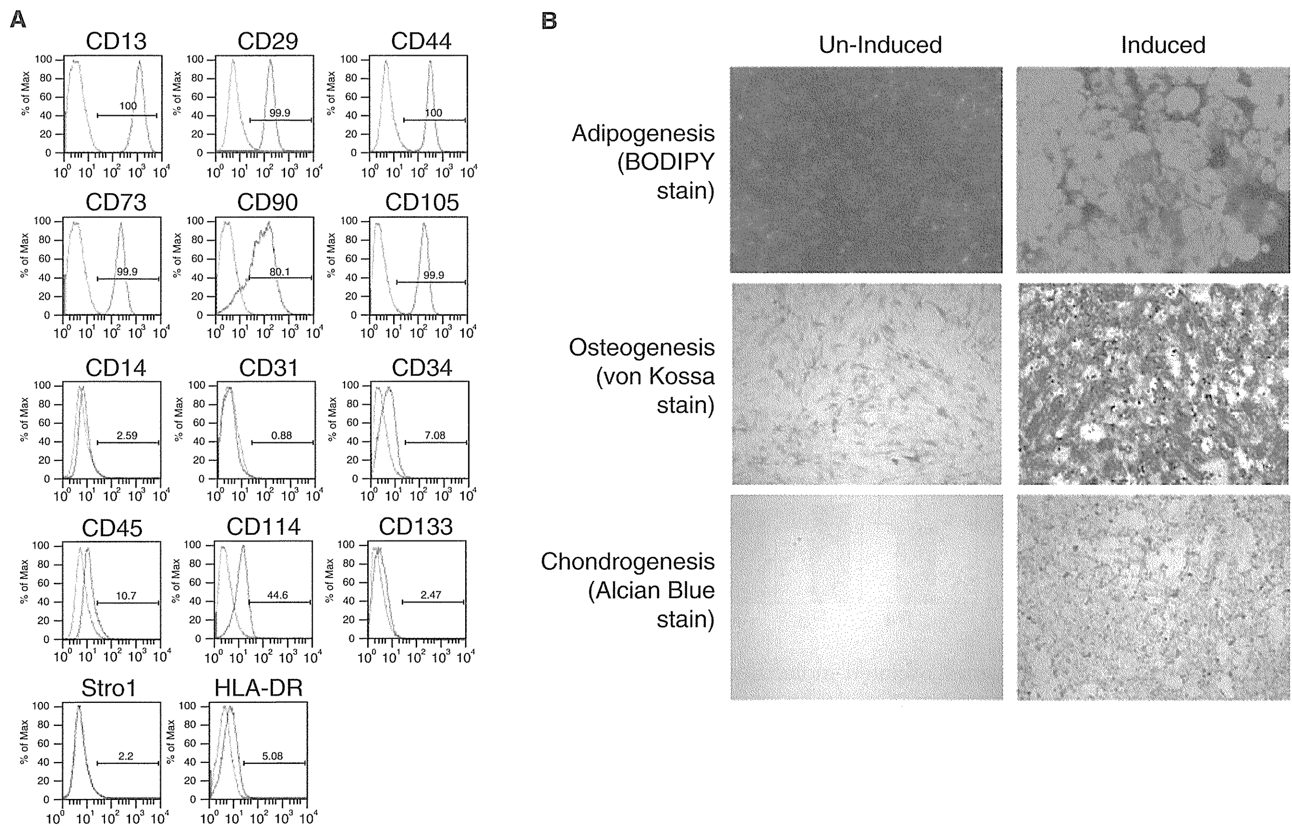

FIG. 1. Characterization of hLASCs. Cell surface markers of hLASCs were analyzed by flow cytometry. (**A**) Representative histograms are demonstrated in blue lines and the respective isotype controls are shown as red lines. CD13, CD29, CD44, CD73, CD90, and CD105 were positive, while CD14, CD31, CD34, CD45, CD117, CD133, Stro1, and HLA-DR were negative. (**B**) In hLASCs, adipogenic, chondrogenic, and osteogenic differentiation could be induced as examined by BODIPY staining, von Kossa staining, or Alician blue staining. Abbreviation: hLASCs, human adipose tissue–derived stromal cells cultured in low serum (containing 2% FBS and FGF-2) medium.

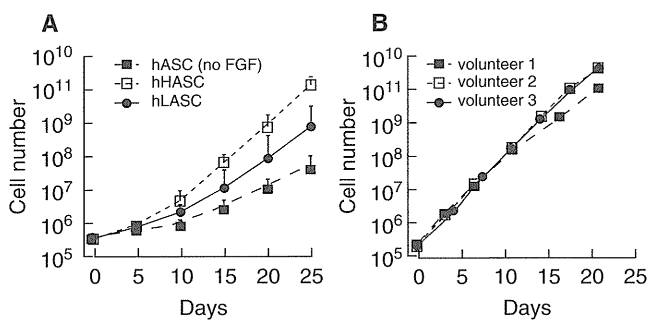

FIG. 2. Growth speeds of hASCs cultured under three different conditions. (**A**) Human HASCs grew the fastest, followed by hLASCs. The growth rate of hASCs (no FGF) was the slowest. (**B**) Human ASCs cultured in a low-serum culture medium using human serum proliferated at a rate which compares favorably to FBS. Abbreviations: hASCs, human adipose tissue–derived stromal cells; hLASCs, hASCs cultured in low serum (containing 2% FBS and FGF-2) medium; hHASCs, hASCs cultured in high serum (containing 20% FBS and FGF-2) medium; hASCs (no FGF), hASCs cultured in conventional (containing 20% FBS only) medium.

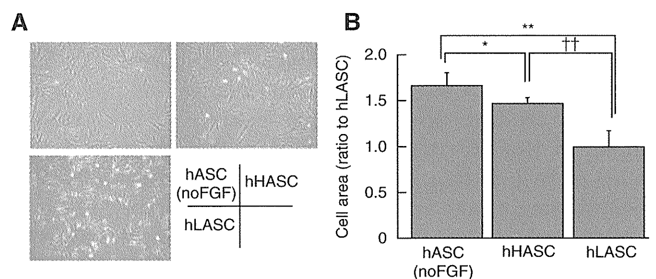

FIG. 3. Cell morphology of hASCs cultured under different conditions. (**A**) Human LASCs were small and round while hHASCs or hASCs (no FGF) were relatively large and flat. Cell areas of hHASCs were significantly larger than those of hLASCs ($^{*}P < 0.05$ vs. hLASCs). (**B**) Cell areas of hASCs (no FGF) were even larger ($^{**}P < 0.01$ vs. hLASCs, $^{\dagger\dagger}P < 0.01$ vs. hHASCs). Abbreviations: hASCs, human adipose tissue–derived stromal cells; hLASCs, hASCs cultured in low serum (containing 2% FBS and FGF-2) medium; hHASCs, hASCs cultured in high serum (containing 20% FBS and FGF-2) medium; hASCs (no FGF), hASCs cultured in conventional (containing 20% FBS only) medium.

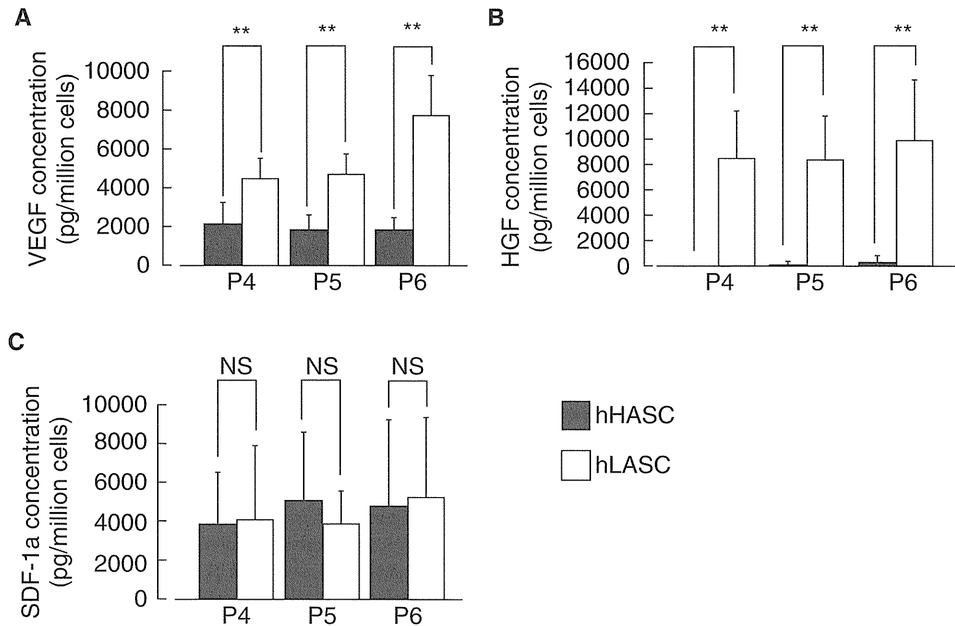

FIG. 4. Secretion of angiogenesis-related molecules by hASCs. The levels of (**A**) VEGF and (**B**) HGF were significantly higher in the hLASC-conditioned medium than those in the hHASC-conditioned medium (**$P < 0.01$). (**C**) Both hLASCs and hHASCs secreted SDF-1α at similar levels. Abbreviations: hASCs, human adipose tissue–derived stromal cells; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; hLASCs, hASCs cultured in low serum (containing 2% FBS and FGF-2) medium; hHASCs, hASCs cultured in high serum (containing 20% FBS and FGF-2) medium; SDF-1α, stromal cell–derived factor 1α.

conditioned media on human EC. The hHASC-conditioned medium significantly increased the number of EC as compared to the control medium. The hLASC-conditioned medium further enhanced EC proliferation (Fig. 5A). In order to examine the effects of VEGF and HGF in the conditioned media on EC proliferation, an experiment using VEGF- and HGF-neutralizing polyclonal antibodies was performed. Adding an anti-HGF antibody to the hLASC-conditioned medium resulted in 33% inhibition, while an anti-VEGF antibody resulted in 54% inhibition. When both antibodies were applied together, greater inhibition (77%) occurred (Fig. 5B).

While EC cultured in hHASC-conditioned media showed significantly lower apoptosis rates versus EC in basal media, those in hLASC-conditioned media showed even lower rates ($P < 0.01$) (Fig. 5C and D). Treatment with anti-HGF neutralizing antibody decreased the antiapoptotic effect of LhASC-conditioned media by 46%, and treatment with anti-VEGF neutralizing antibody by 52%. When both antibodies were applied together, an even greater decrease (70%) was attained (Fig. 5E).

In vivo therapeutic effects of hHASCs and hLASCs on rat hindlimb ischemia

Laser Doppler analyses of blood flow. The effects of hASCs were studied by using laser Doppler blood flowmetry at days 0, 7, and 14. The ratio of ischemic/normal hindlimb blood flow had significantly increased in the hHASC-treated rats as compared with the control group ($P < 0.05$). The blood flow ratio was further improved by the administration of hLASCs (Fig. 6A).

Capillary densities in the hindlimbs. The thigh muscle sections were stained for CD31, and the capillaries were stained in dark red. By day 14, hHASCs had increased the ratio of capillary/muscle fiber by 1.25-fold as compared with media only. An even better result was obtained by the administration of hLASCs, 1.44-fold increase compared to media only (Fig. 6B).

Concentrations of VEGF, HGF, and SDF-1α in the muscles. In order to further study the mechanisms, the levels of the angiogenic factors, VEGF, HGF, and SDF-1α, known to be produced by hASCs in vitro, were measured using the homogenates of the muscles obtained at day 3. The level of human VEGF was significantly higher in the hLASC-treated group as compared with the hHASC-treated group. (Fig. 7A and B). The level of human HGF in the hLASC group was 9.7 times higher than that in the HhASCs group. The SDF-1α contents were increased by the administration of hHASCs or hLASCs. However, no difference was observed between these two (Fig. 7C).

Involvement of hASCs in angiogenesis. At day 7 after cell transplantation, many human Lamin A/C-positive cells were observed around the injected area. However, they were not incorporated into the EC layers, which were positive for CD31. Nor were they identified in the smooth muscle layers, which were positive for α-smooth muscle actin (data not shown).

Discussion

Recently, stem/progenitor cells of mesenchymal lineage have been discovered in fat tissue [17,32]. These ASC can be easily expanded and differentiated into variety of cells

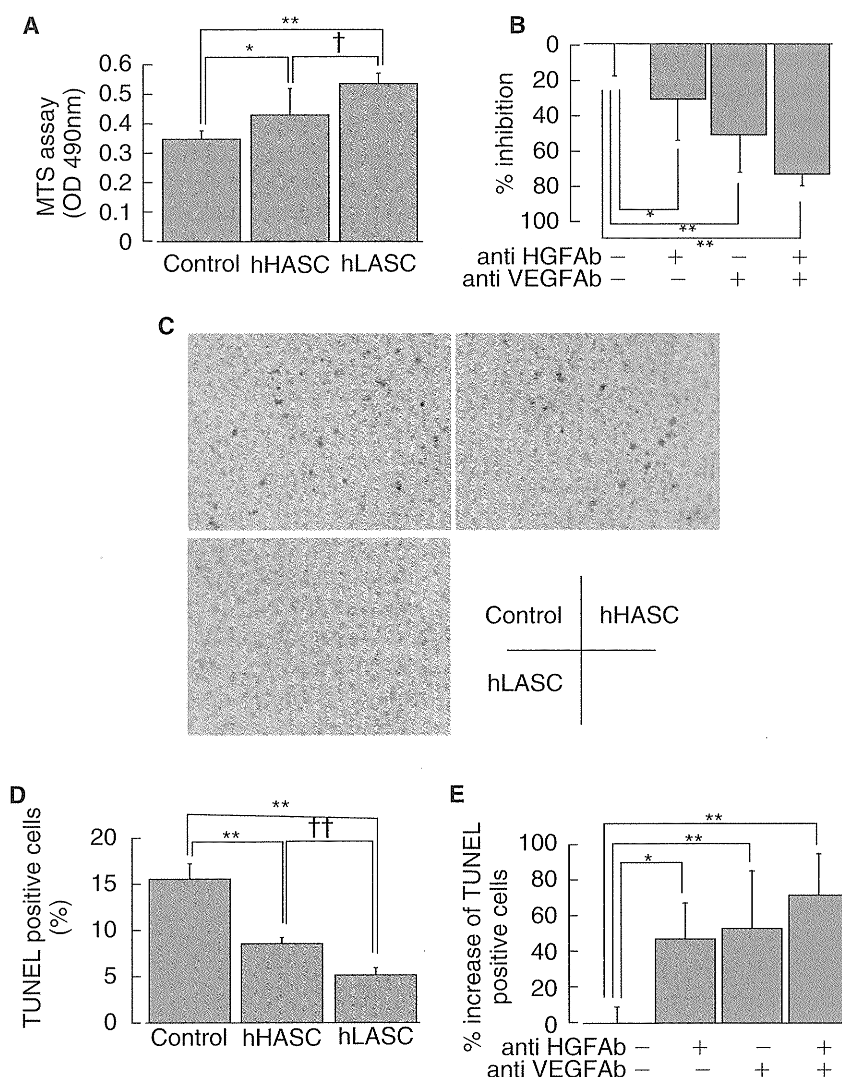

FIG. 5. Effects of hHASC- and hLASC-conditioned media on EC bioactivities. (**A**) MTS assay demonstrated that hHASC-conditioned medium significantly increased the number of ECs as compared to the control medium (*$P < 0.05$ vs. control). The hLASC-conditioned medium further enhanced EC proliferation (**$P < 0.01$ vs. control, †$P < 0.05$ vs. hHASCs). (**B**) VEGF- and/or HGF-neutralizing polyclonal antibodies were added to the hLASC-conditoned medium and MTS assay was performed. Both of these antibodies significantly inhibited EC proliferation, and even greater inhibition occurred when applied together (*$P < 0.05$ vs. control, **$P < 0.01$ vs. control). (**C** and **D**) TUNEL assay demonstrated that ECs cultured in hHASC-conditioned media showed significantly lower apoptosis rates versus ECs in basal media (**$P < 0.01$ vs. control), while those in hLASC-conditioned media showed even lower rates (**$P < 0.01$ vs. control, ††$P < 0.01$ vs. hHASCs). Treatment with either anti-HGF neutralizing antibody or anti-VEGF neutralizing antibody decreased the antiapoptotic effect of LhASC-conditioned media. (**E**) When both antibodies were applied together, an even greater decrease was attained (*$P < 0.05$ vs. control, **$P < 0.01$ vs. control). Abbreviations: hHASCs, human adipose tissue–derived stromal cells cultured in high serum (containing 20% FBS and FGF-2) medium; hLASCs, human adipose tissue–derived stromal cells cultured in low serum (containing 2% FBS and FGF-2) medium; EC, endothelial cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolim, inert salt; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

along not only mesenchymal lineage but also along hematopoietic [33] and neuronal [34] lineages. Since adipose tissue is abundant, and can be obtained repeatedly under local anesthesia with a minimum of patient discomfort, hASCs have great potential as an autologous cell source for regenerative therapy. In order to better apply hASCs to the patients, it is crucial to eliminate all reagents of animal origin without hampering the efficiency of cell expansion.

In this study, we successfully established a simple and efficient expansion system of MSCs from human adult adipose tissue. With a low serum culture system, we produced 1×10^8 of hLASC tissue within 3 weeks by culturing

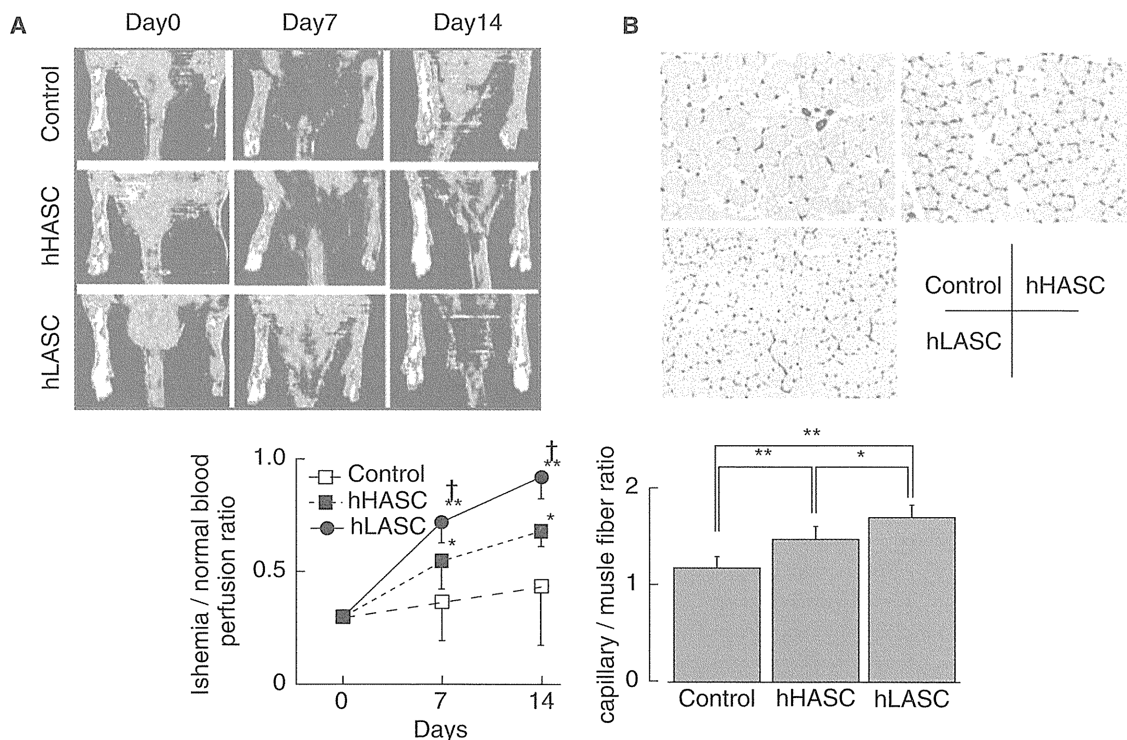

FIG. 6. Effects of hASCs injection in rat ischemic hindlimb model. (**A**) Laser Doppler blood flowmetry were performed at Days 0, 7, and 14. The ratio of ischemic/normal hindlimb blood flow had significantly increased in the hHASC-treated rats as compared with the control group (*$P < 0.05$). Administration of hLASCs further improved the blood flow (**$P < 0.01$ vs. control, $^{\dagger}P < 0.05$ vs. hHASCs). (**B**) The thigh muscle sections were stained for CD31. By Day 14, hHASCs had increased the ratio of capillary/muscle fiber by 1.25-fold as compared with vehicle only (**$P < 0.01$ vs. control). An even better result was obtained by the administration of hLASCs (**$P < 0.01$ vs. control, $^{\dagger}P < 0.05$ vs. hHASCs). Abbreviations: hASCs, human adipose tissue–derived stromal cells; hHASCs, hASCs cultured in high serum (containing 20% FBS and FGF-2) medium; hLASCs, hASCs cultured in low serum (containing 2% FBS and FGF-2) medium.

the SVF cells obtained from 1 g of tissue in a medium containing 2% FBS and FGF-2. Using FGF-2, a better cell expansion rate was obtained in a low (2%) serum culture than with conventional media (20% serum without FGF-2). Previous reports have already demonstrated that hASCs can be expanded using human serum instead of FBS [35,36]. However, in order to reduce the concentration of human serum, a special medium containing various growth factors and cytokines has been employed [36]. In our method, the only essential growth factor is human FGF-2. Previous reports have demonstrated that FGF-2 maintains or enhances the multilineage differentiation potential of MSCs [37,38], increases the proliferation rate of MSCs [39], and suppresses the cellular senescence of MSCs [40]. Of note is the fact that FGF-2 has been used for patients to promote tissue repair without major adverse effects [41]. We then confirmed that human serum could substitute for FBS. By calculation, 60 mL of human serum is required to obtain 1×10^9 of hLASCs, whereas >10 times the volume of serum is needed to get the same number of hASCs when conventional media is used.

FACS analysis revealed that hLASCs have cell surface markers indistinguishable from those on BM-MSCs or hASCs cultured in a conventional medium. We also found that like BM-MSCs, hLASCs can be differentiated into cells of mesenchymal lineage, indicating that hLASCs can be considered MSCs. Nonetheless, the morphology of hLASCs was clearly different from that of hHASCs. The former were small, round, and thick while the latter were relatively large and flat. A recent report demonstrated that MSCs which had thicker cytoplasm proliferated better and were more resistant to senescence than those with thinner cytoplasm [42]. These findings indicate that hLASCs and hHASCs may have different bioactivities.

We then studied the levels of VEGF and HGF in the conditioned media of hASCs. These two molecules have been shown to play important roles in angiogenesis and tissue regeneration, and are known to be secreted by hASCs [43]. To our surprise, hLASCs secreted a significantly higher amount of VEGF than hHASCs. Moreover, hLASCs secreted HGF at a high level, while hHASCs secreted much less. The levels of SDF-1α, a molecule which has been shown to recruit EPC, were not different between these two. We also studied the effects of the conditioned media from hLASCs and hHASCs on EC. We found that hLASC-conditioned media enhanced EC proliferation and inhibited EC apoptosis more efficiently than did hHASC-conditioned media. Antibodies against VEGF and HGF almost, but not completely, negated these effects. The results suggest that VEGF and HGF are two major cytokines promoting the repair of vessels and protecting them from injury although other molecules may still play important roles.

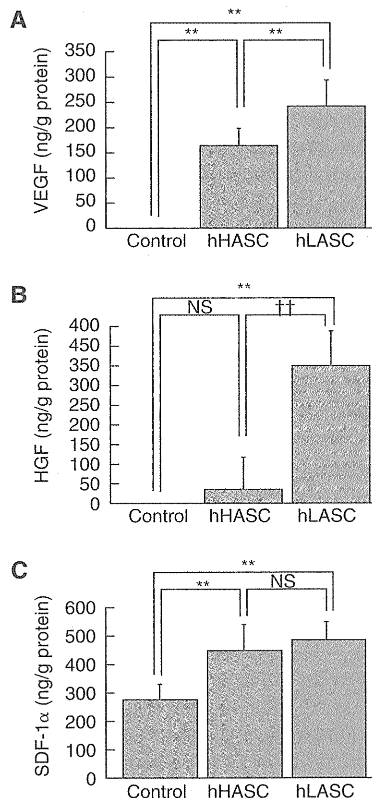

FIG. 7. Levels of angiogenic factors in the muscles of ischemic limbs obtained at day 3. (**A**) The level of human VEGF was significantly higher in the hLASC-treated group as compared with the hHASC-treated group (**$P < 0.01$ vs. Control, ††$P < 0.01$ vs. hHASCs). (**B**) The level of human HGF in the hLASC group was 9.7 times higher than that in the hHASCs group (**$P < 0.01$ vs. Control, ††$P < 0.01$ vs. hHASCs). (**C**) The SDF-1α contents were increased by the administration of hHASCs or hLASCs. However, no difference was observed between these two. Abbreviations: VEGF, vascular endothelial growth factor; hLASCs, human adipose tissue–derived stromal cells cultured in low serum (containing 2% FBS and FGF-2) medium; hHASCs, human adipose tissue–derived stromal cells cultured in high serum (containing 20% FBS and FGF-2) medium; HGF, hepatocyte growth factor; SDF-1α, stromal cell–derived factor 1α.

To study the in vivo therapeutic potential, we transplanted hASCs to rats with hindlimb ischemia. At 2 weeks after transplantation, hLASCs had improved blood flow, increased capillary density and raised the levels of human VEGF and human HGF in the muscle tissue as compared with hHASCs. Immunohistochemistry revealed, however, that transplanted cells were not incorporated into blood vessels. Two different hypotheses have been postulated concerning how stem/progenitor cells restore tissue function. One is that administered cells act as integrated participants in the target tissue, and the other is that they are not incorporated into a target tissue but deliver multiple secretory molecules, possibly complementary angiogenic and antiapoptotic growth factors. In BM transplantation for hematological disorder, hematopoietic stem cells work through the first mechanism. In BM stem/progenitor cell therapy for ischemic hearts or legs, the mechanism remains unproven [2,44,45]. Concerning adipose-derived stem/progenitor cells, both mechanisms have been demonstrated [46–49]. The results of this study indicate that hLASCs act on the ischemic tissue by the latter mechanism, in other words multi-cytokine secretion.

Our finding that transplanted hLASCs were not differentiated into vascular cells may be attributed to the protocol employed in this study, that human cells were transplanted to rat. We might underestimate the true potential of hLASCs because of some minor rejection and also because of the bioincompatibility of molecules between different species. Nonetheless, nonincorporation of the transplanted cells should not necessarily be regarded as a limitation of hLASC therapy. Rather, it can be an advantage since carcinogenesis of the transplanted cells has been a concern in stem cell therapy. Now, we are preparing a clinical trial to transplant hLASCs to the ischemic legs of patients with severe peripheral artery disease. This kind of clinical study will prove the true potential of hLASCs.

In conclusion, we established a novel low serum culture system for hASCs. Unexpectedly, hLASCs showed clear advantages over hHASCs, secreting higher levels of angiogenic and antiapoptotic growth factors, and improving hindlimb ischemia. hLASCs may have great potential in regenerative cell therapy for damaged organs in the clinical setting.

Acknowledgments

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