Optimization of culture conditions for endothelial progenitor cells from porcine bone marrow *in vitro*

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Abstract

Objectives: The aim of this study was to determine an optimal culture method for porcine bone marrow-derived endothelial progenitor cells (EPCs).

Materials and methods: Mononuclear cells (MNCs) were isolated by density centrifugation and differentiated into EPCs *in vitro*. At first-passage, EPCs were cultured at different cell densities (5×10^3 , 1×10^4 , 2×10^4 or $5 \times 10^4/\text{cm}^2$) and in basic medium (EGM, medium 199, DMEM or 1640) supplemented with FBS (2%, 5%, 10% or 20%) and different combinations of cytokines (VEGF, VEGF + bFGF, VEGF + bFGF + EGF, or VEGF + bFGF + EGF + IGF), the experiment being based on L_{64} (4^{21}) orthogonal design.

Results and conclusions: This demonstrated that the optimal culture method for our EPCs displayed higher expansion and migration rates as compared to other groups, by analysis of variance; that is, cultured at $1 \times 10^4/\text{cm}^2$ in M199 supplemented with 10% FBS and VEGF + bFGF + IGF + EGF. Furthermore, percentage of positive cells stained by Dil-ac-LDL and FITC-UEA-1 was more than 65%, and as shown by immunohistochemistry, these cells also stained positively for CD133, CD34 and KDR. The present study indicates that the number and function of porcine EPCs significantly increased when using our optimized culture parameters.

Introduction

Since 1997, postnatal vasculogenesis has been reported to be an important mechanism for neoangiogenesis, through

marrow-derived circulating endothelial progenitor cells (EPCs) (1). Emerging evidence indicates that EPCs play an important role in adult vasculogenesis in response to physiological and pathological stimuli (2,3). EPCs can ameliorate the function of ischaemic organs, possibly by both induction and modulation of vasculogenesis by stimulating re-endothelialization of injured blood vessels (4).

Accordingly, more recent research has shifted to the therapeutic application of endothelial cells for atherosclerotic disease and its peripheral and cardiovascular complications (5). In particular, EPCs for these applications may revolutionize therapy, as a result of the respective population growth potential of these cells (6). EPCs have been implicated in myocardial repair after infarction, in propagation of angiogenesis following ischaemia, and in vascular repair after injury (7). Furthermore, there is increasing evidence regeneration of heart and vasculature is governed by EPCs. Most importantly, *in vitro* culture of EPCs is subsequently applied to patients as a cell-based therapy (8,9), or is used for extensive experimental analysis (10).

Isolation of putative EPCs provides a powerful tool, not only for basic research of differentiation pathways but also for therapeutic application with respect to replacement of pathologically altered vessels (11). However, therapeutic effects of EPCs in clinical trials are restricted compared to those of animal experimental studies due to limited numbers of EPCs. In addition, growing evidence suggests that smoking, hypercholesterolaemia, diabetes mellitus and advanced age could be risk factors for reduction in number and function of EPCs (12,13). Moreover, reduction in function and number of EPCs contribute to the limited effects of angiogenesis in patients and experimental animals (14).

Interestingly, cell culture conditions may be able to enhance cell function and increase cell number through *in vitro* manipulation. *Ex vivo* culture and expansion of EPCs could be a promising strategy to overcome the clinical problem of limited cell number. Although EPCs have

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been successfully isolated from porcine bone marrow, the resultant number of cells is limited and EPC culture methods have not been standardized (15). Considerable time is required in *in vitro* culture to establish an optimal method, but this manipulation of optimization remains unclear because factors affecting culture of EPCs are complicated. Therefore, it has been our purpose to optimize culture conditions for EPCs from porcine bone marrow, to increase number of functionally active EPCs for transplantation.

Materials and methods

Porcine bone marrow

All animals received care in compliance with Ministry of Science and Technology of China 'Guide for the care and use of laboratory animals' and 'Principle of laboratory animal care' (NIH publication No.86-23, revised 1985). All components of the experiments were approved by our institutional ethics committee. Bone marrow was obtained from healthy mini pigs. All pigs (approximately 20 kg, $n = 9$) were anaesthetized with 10 mg/kg ketamine (Ziyuanpharm, Shanghai, China) and 5 mg/kg thiopental (Ziyuanpharm) and were maintained in a gas mixture of oxygen at 1.5–2.0 l/min and isoflurane (Ziyuanpharm) at 0.75–3.0%. Bone marrow cells were aspirated from the ilium under sterile conditions and were anti-coagulated with 0.05 mg/ml heparin (Ziyuanpharm). Cell separation was performed within 30 min of marrow collection.

Orthogonal design

According to previous studies (8,10,11), we chose four main factors with four levels each (Table 1); these cells were divided into 64 groups based on $L_{64}(4^{21})$ orthogonal design (Table 2), which can reduce experimental times, revealing the interactive effects of factors without affecting the result of the study.

Table 1. Factor-level for orthogonal design

Level	Factor			
	Culture density (/cm ²)	Basic medium	FBS concentration (%)	Cytokines
1	5×10^3	EGM-2	2	VEGF
2	1×10^4	M199	5	VEGF + bFGF
3	2×10^4	DMEM	10	VEGF + bFGF + EGF
4	5×10^4	1640	20	VEGF + bFGF + EGF + IGF

Cell isolation and culture

Mononuclear cells (MNCs) were isolated from the bone marrow by density-gradient centrifugation (IEC, Chicago, IL, USA) with Ficoll (Sigma, Ronkonkoma, NY, USA). Immediately after isolation, MNCs ($1 \times 10^6/\text{cm}^2$) were plated in six-well culture dishes (Nunc, Rochester, MI, USA) coated with 20 µg/ml of fibronectin (Chemicon, Temecula, CA, USA) and were maintained in endothelial growth medium-2 (EGM-2) (Invitrogen, Frankfurt StraÙe, Germany) supplemented with 20% foetal bovine serum (FBS) (Invitrogen) and vascular endothelial growth factors (VEGF, 10 ng/ml) (Peprotech, London, UK). The cells were cultured in an incubator (ThermoForma, Marietta, OH, USA) at 37 °C and 5% CO₂. After 48 h culture, non-adherent cells were removed by washing with phosphate buffered saline (PBS). Medium was subsequently replaced every 3 days, and primary culture of MNCs differentiating into EPCs was maintained for 7 days (16). First-passage EPCs were cultured at different cell densities: 5×10^3 , 1×10^4 , 2×10^4 or $5 \times 10^4/\text{cm}^2$ in a basic medium: EGM-2, medium 199 (M199), Dulbecco's modified Eagle's medium (DMEM), or 1640 supplemented with FBS (2%, 5%, 10% or 20%), and in different combinations of cytokines: VEGF (10 ng/ml), VEGF + basic fibroblast growth factor (bFGF, 50 ng/ml) (Peprotech), VEGF + bFGF + epidermal growth factor (EGF, 20 ng/ml) (Peprotech), or VEGF + bFGF + EGF + insulin-like growth factor (IGF, 50 ng/ml) (Peprotech). In addition, our culture medium also contained 200 ng/ml hydrocortisone, 1.0 µg/ml ascorbic acid, 0.1 mg/ml penicillin, 0.1 mg/ml streptomycin, 50 ng/ml amphotericin B and 0.05 mg/ml heparin (Ziyuanpharm).

Expansion assay

Expansion of every cell group was assayed by counting expansion multiples (number of fourth passage viable cells/number of first passage viable cells). First, first-passage cells (Table 2) were reseeded into 24-well flat-bottomed plates (Nunc) with 1 ml medium (Table 2). These were then incubated at 37 °C in 5% CO₂ for 72 h, then all groups of cells were detached with trypsin–EDTA (Invitrogen) and centrifuged. Finally, number of viable cells was manually counted using a haematological counter beneath a phase-contrast microscope (Olympus, Tokyo, Japan), and non-viable cells were distinguished by utilization of 4% trypan blue stain (Invitrogen).

Migration assay

After 3 days culture, first-passage cells were harvested, washed twice in PBS and resuspended in EGM-2. A

Table 2. $L_{64}(4^{21})$ orthogonal design group and results (mean $\pm$ SD)

Group	Density (/cm ²)	Medium (/ml)	FBS (/ml)	Cytokines (Ng/ml)	Expansion passage 1–4	Migration rate (%)	Double-positive staining (%)	Immunohistochemistry		
								CD133	CD34	KDR
1	5×10^3	DMEM	10%	VEGF + bFGF + EGF + IGF	47.17 $\pm$ 2.23	15.01 $\pm$ 0.67	88.53 $\pm$ 3.84	++	++	+++
2	5×10^3	DMEM	5%	VEGF + bFGF + EGF + IGF	31.33 $\pm$ 1.01	15.13 $\pm$ 0.51	86.93 $\pm$ 3.66	+	++	+++
3	5×10^3	1640	5%	VEGF + bFGF	5.74 $\pm$ 0.22	9.83 $\pm$ 0.48	77.19 $\pm$ 2.98	+	++	++
4	5×10^3	EGM-2	5%	VEGF	19.25 $\pm$ 0.91	9.93 $\pm$ 0.45	78.37 $\pm$ 2.77	+	++	++
5	1×10^4	1640	5%	VEGF + bFGF + EGF	6.54 $\pm$ 0.30	10.45 $\pm$ 0.50	79.20 $\pm$ 3.34	+	++	++
6	1×10^4	DMEM	2%	VEGF	16.58 $\pm$ 0.70	9.78 $\pm$ 0.39	73.19 $\pm$ 2.72	+	++	++
7	1×10^4	EGM-2	5%	VEGF + bFGF + EGF + IGF	69.14 $\pm$ 3.15	17.09 $\pm$ 0.82	89.77 $\pm$ 3.99	++	++	+++
8	1×10^4	EGM-2	2%	VEGF + bFGF + EGF + IGF	60.83 $\pm$ 2.57	17.11 $\pm$ 0.77	86.49 $\pm$ 3.85	++	++	+++
9	2×10^4	DMEM	10%	VEGF + bFGF + EGF	43.03 $\pm$ 1.64	13.91 $\pm$ 0.65	82.33 $\pm$ 3.43	+	++	+++
10	2×10^4	DMEM	5%	VEGF + bFGF + EGF	38.89 $\pm$ 1.55	13.78 $\pm$ 0.66	80.56 $\pm$ 2.85	+	++	+++
11	2×10^4	M199	2%	VEGF + bFGF + EGF + IGF	65.56 $\pm$ 2.67	15.34 $\pm$ 0.78	86.49 $\pm$ 3.94	+	++	+++
12	2×10^4	1640	10%	VEGF	4.75 $\pm$ 0.21	8.95 $\pm$ 0.49	68.22 $\pm$ 2.18	+	++	++
13	5×10^4	1640	2%	VEGF + bFGF + EGF + IGF	4.19 $\pm$ 0.19	10.37 $\pm$ 0.51	75.39 $\pm$ 2.72	+	++	++
14	5×10^4	EGM-2	20%	VEGF + bFGF + EGF	31.78 $\pm$ 1.44	10.29 $\pm$ 0.48	74.91 $\pm$ 2.38	+	++	++
15	5×10^4	DMEM	5%	VEGF + bFGF	10.65 $\pm$ 0.57	8.81 $\pm$ 0.42	68.97 $\pm$ 2.88	+	++	++
16	5×10^4	DMEM	10%	VEGF	13.11 $\pm$ 0.58	8.98 $\pm$ 0.39	70.27 $\pm$ 2.19	+	++	++
17	5×10^3	M199	2%	VEGF + bFGF + EGF	49.27 $\pm$ 2.33	14.92 $\pm$ 0.74	88.35 $\pm$ 3.49	+	++	+++
18	5×10^3	DMEM	20%	VEGF + bFGF + EGF + IGF	47.51 $\pm$ 1.98	16.10 $\pm$ 0.79	89.25 $\pm$ 4.17	+	++	+++
19	5×10^3	EGM-2	2%	VEGF	17.01 $\pm$ 0.80	9.87 $\pm$ 0.47	75.74 $\pm$ 3.16	+	++	++
20	5×10^3	EGM-2	10%	VEGF	22.79 $\pm$ 1.05	10.69 $\pm$ 0.51	80.54 $\pm$ 2.99	+	+	++
21	1×10^4	M199	2%	VEGF + bFGF	29.91 $\pm$ 1.42	13.66 $\pm$ 0.67	82.45 $\pm$ 3.57	+	++	++
22	1×10^4	DMEM	20%	VEGF	20.03 $\pm$ 0.85	10.71 $\pm$ 0.49	84.15 $\pm$ 2.93	+	+	++
23	1×10^4	1640	10%	VEGF + bFGF + EGF	7.67 $\pm$ 0.37	10.87 $\pm$ 0.51	78.54 $\pm$ 3.84	+	++	++
24	1×10^4	DMEM	10%	VEGF	18.30 $\pm$ 0.73	13.75 $\pm$ 0.63	79.45 $\pm$ 3.59	+	++	++
25	2×10^4	1640	2%	VEGF	3.81 $\pm$ 1.43	7.97 $\pm$ 0.34	65.17 $\pm$ 3.23	+	+	++
26	2×10^4	M199	20%	VEGF + bFGF + EGF + IGF	87.71 $\pm$ 4.35	17.48 $\pm$ 0.79	89.53 $\pm$ 4.04	+	++	+++
27	2×10^4	DMEM	2%	VEGF + bFGF + EGF	34.19 $\pm$ 1.32	13.41 $\pm$ 0.65	79.56 $\pm$ 2.99	+	++	+++
28	2×10^4	M199	5%	VEGF + bFGF + EGF + IGF	72.37 $\pm$ 2.45	16.46 $\pm$ 0.81	89.48 $\pm$ 3.96	+	++	+++
29	5×10^4	M199	5%	VEGF	13.13 $\pm$ 0.71	9.03 $\pm$ 0.45	74.31 $\pm$ 2.65	+	++	++
30	5×10^4	M199	10%	VEGF	15.26 $\pm$ 0.72	9.45 $\pm$ 0.48	79.13 $\pm$ 2.87	+	++	++
31	5×10^4	EGM-2	5%	VEGF + bFGF + EGF	24.78 $\pm$ 1.31	10.25 $\pm$ 0.47	80.28 $\pm$ 2.55	+	++	++
32	5×10^4	1640	10%	VEGF + bFGF + EGF + IGF	5.45 $\pm$ 0.26	9.29 $\pm$ 0.46	71.27 $\pm$ 2.51	+	++	++
33	5×10^3	1640	10%	VEGF + bFGF	6.74 $\pm$ 0.25	9.99 $\pm$ 0.49	73.18 $\pm$ 2.88	+	++	+++
34	5×10^3	DMEM	2%	VEGF + bFGF + EGF + IGF	28.41 $\pm$ 1.11	14.53 $\pm$ 0.61	87.39 $\pm$ 3.78	+	++	+++
35	5×10^3	1640	2%	VEGF + bFGF	6.23 $\pm$ 0.24	9.66 $\pm$ 0.43	75.18 $\pm$ 2.87	+	++	++
36	5×10^3	M199	10%	VEGF + bFGF + EGF	68.17 $\pm$ 2.99	15.89 $\pm$ 0.78	86.34 $\pm$ 3.89	+	++	+++

Table 2. (Continued)

Group	Density (/cm ²)	Medium (/ml)	FBS (/ml)	Cytokines (Ng/ml)	Expansion passage 1-4	Migration rate (%)	Double-positive staining (%)	Immunohistochemistry		
								CD133	CD34	KDR
37	1 × 10 ⁴	EGM-2	5%	VEGF + bFGF	29.11 ± 1.34	13.11 ± 0.59	75.56 ± 2.92	+	++	++
38	1 × 10 ⁴	EGM-2	20%	VEGF + bFGF + EGF + IGF	94.75 ± 2.72	17.10 ± 0.83	90.77 ± 3.94	++	+++	+++
39	1 × 10 ⁴	DMEM	10%	VEGF + bFGF	20.05 ± 0.92	12.97 ± 0.58	75.34 ± 2.89	+	++	+++
40	1 × 10 ⁴	M199	10%	VEGF + bFGF + EGF + IGF	131.22 ± 9.5	20.08 ± 0.93	92.59 ± 4.44	++	+++	+++
41	2 × 10 ⁴	1640	5%	VEGF	4.10 ± 0.18	8.11 ± 0.35	69.78 ± 2.24	+	+	++
42	2 × 10 ⁴	DMEM	20%	VEGF + bFGF + EGF	46.14 ± 1.73	13.51 ± 0.66	78.23 ± 3.05	+	++	+++
43	2 × 10 ⁴	EGM-2	5%	VEGF + bFGF	13.57 ± 0.63	13.15 ± 0.62	77.32 ± 2.87	+	++	+++
44	2 × 10 ⁴	EGM-2	10%	VEGF + bFGF	15.37 ± 0.71	13.12 ± 0.60	78.57 ± 2.90	+	++	+++
45	2 × 10 ⁴	EGM-2	2%	VEGF + bFGF + EGF	14.38 ± 0.55	10.11 ± 0.45	79.28 ± 2.51	+	++	++
46	5 × 10 ⁴	M199	20%	VEGF	16.07 ± 0.77	9.50 ± 0.48	69.83 ± 2.81	+	++	++
47	5 × 10 ⁴	M199	2%	VEGF	11.63 ± 0.54	8.77 ± 0.43	70.39 ± 2.51	+	+	++
48	5 × 10 ⁴	1640	5%	VEGF + bFGF + EGF + IGF	4.82 ± 0.21	9.20 ± 0.45	67.27 ± 2.51	+	++	++
49	5 × 10 ³	M199	5%	VEGF + bFGF + EGF	53.85 ± 1.76	14.97 ± 0.72	84.59 ± 3.41	+	++	+++
50	5 × 10 ³	EGM-2	20%	VEGF	23.86 ± 1.11	10.78 ± 0.53	73.71 ± 3.46	+	++	++
51	5 × 10 ³	1640	20%	VEGF + bFGF	6.71 ± 0.26	10.59 ± 0.51	74.18 ± 2.56	+	++	++
52	5 × 10 ³	M199	20%	VEGF + bFGF + EGF	72.19 ± 2.34	15.92 ± 0.77	87.58 ± 4.15	+	++	+++
53	1 × 10 ⁴	M199	20%	VEGF + bFGF	73.18 ± 2.33	14.54 ± 0.69	82.45 ± 3.53	+	++	+++
54	1 × 10 ⁴	1640	20%	VEGF + bFGF + EGF	8.05 ± 0.31	11.18 ± 0.54	83.54 ± 3.75	+	++	++
55	1 × 10 ⁴	1640	2%	VEGF + bFGF + EGF	5.84 ± 0.21	9.89 ± 0.49	80.51 ± 3.42	+	++	++
56	1 × 10 ⁴	M199	5%	VEGF + bFGF	38.24 ± 1.72	13.60 ± 0.65	81.41 ± 3.53	+	++	++
57	2 × 10 ⁴	1640	20%	VEGF	4.99 ± 0.21	8.21 ± 0.41	66.98 ± 2.27	+	++	++
58	2 × 10 ⁴	M199	10%	VEGF + bFGF + EGF + IGF	93.12 ± 4.19	18.01 ± 0.89	90.51 ± 4.42	+	++	+++
59	2 × 10 ⁴	EGM-2	20%	VEGF + bFGF	16.36 ± 0.58	13.45 ± 0.62	76.32 ± 2.97	+	++	+++
60	2 × 10 ⁴	EGM-2	2%	VEGF + bFGF	9.98 ± 0.47	12.81 ± 0.63	74.35 ± 2.64	+	++	+++
61	5 × 10 ⁴	1640	20%	VEGF + bFGF + EGF + IGF	7.18 ± 0.30	9.88 ± 0.48	68.25 ± 2.55	+	++	++
62	5 × 10 ⁴	DMEM	20%	VEGF + bFGF	14.79 ± 0.64	9.14 ± 0.45	69.23 ± 2.25	+	++	++
63	5 × 10 ⁴	EGM-2	10%	VEGF + bFGF + EGF	29.53 ± 1.39	11.22 ± 0.52	70.03 ± 2.51	+	++	++
64	5 × 10 ⁴	DMEM	2%	VEGF + bFGF	9.01 ± 0.43	8.49 ± 0.41	65.78 ± 2.28	+	+	++

modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) was used, with polycarbonate filter, pore size of 8 µm (Neuro Probe). This was placed between upper and lower chambers and EPCs (Table 2) were positioned in the upper chamber, the lower chamber was filled with medium (Table 2). The chamber was incubated at 37 °C with 5% CO₂ for 12 h; cells that had migrated were found on the lower side of the filter. These were washed in PBS, fixed in methanol (Ziyuanpharm) and stained with Giemsa stain (Sigma). In every group, migrational activity was evaluated by counting numbers of cells (×40) in respective fields.

Immunohistochemistry

Glass slides of cells (Nunc) were air-dried and immunostained for expression CD133, CD34 and vascular endothelial growth factor receptor 2 (VEGFR-2, KDR). Slides were placed in Petri dishes coated with fibronectin package at 4 °C for 24 h; they were then fixed in formalin (Ziyuanpharm) for 10 min, dropped on slides with Tryting-100 (Sigma). Preparations were incubated in primary antibodies: rabbit anti-goat CD133 (cross-reacting with pig; Santa Cruz, CA, USA), pig anti-goat CD34 (cross-reacting with pig; USA) and mouse anti-pig KDR (Upstate, Charlottesville, VA, USA). Subsequently, they were stained in the corresponding secondary antibody for 30 min at 37 °C and fixed in methanol for 10 min at -20 °C. Sections were incubated at 37 °C for 30 min then washed three times in PBS and were then stained in diaminobenzidine (DAB; Sigma), dehydrated and sealed with neutral gum (Ziyuanpharm). Finally, they were visualized using an Olympus light microscope.

The present study used a dual score semi-quantitative method according to cells the extent of staining and percentage of reacted cells (17,18). On the basis of extent of staining, little or no final reaction product was 0; light was 1 and deep was 2. Percentage of stained cells <5% was 0, 5–25% was 1, 26–50% was 2 and >50% was 3. The concluding score for each group was documented as extent of staining and percentage of stained cells. Score of 0–1 was negative (–), score of 2–3 was low degree positive (+), score of 4 was moderately positive (++) and score of 6 was highly positive (+++).

Fluorescent staining

Fluorescent staining of adherent cells on glass slides was conducted 3 days after first passage cell culture. First, every group of cells was incubated in 12.5 pg Dil-labelled acetylated low-density lipoprotein (Dil-ac-LDL; Invitrogen) diluted 1:20 in EGM-2 at 37 °C for 1 h. Cells were fixed in formalin for 10 min. Then 50 µg of FITC-labelled *Ulex europaeus* agglutinin 1 (FITC-UEA-1; Sigma) was

diluted 1:20 in PBS and applied to slides for 1 h. Finally, double-positive cells were counted manually in five random microscopic fields using confocal laser microscopy (Leica, Allendale, NJ, USA).

Statistical analysis

Statistical analysis was performed using the appropriate statistical package for social science (SPSS, Release 10.0 software, Chicago, IL, USA). Data were expressed as mean ± standard deviation and were analysed using analysis of variance followed by the orthogonal test. $P < 0.05$ was considered as statistically significant.

Results

Morphological characteristics of EPCs

Mononuclear cells were found to be round or oval in shape, none of them being attached during the first 2 days of culture; attached cells were observed as early as 3 day. After 5 days culture, number of attached cells increased then formed colonies (Fig. 1a). After 7 days subsequent to plating, most colonies had reached near confluence. Morphological or population growth characteristics of cells in different culture conditions were found not to be obviously altered.

Immunohistochemical observations

EPCs were considered to be characterized by expression of CD133, CD34 and KDR. Positive cells were stained brown/reddish brown by DAB. In our cultures, every group cells was immunocytochemically positive for CD133, CD34 and KDR, and optimal culture conditions for cells (group 40) showed strongly positive expression CD133 (++), CD34 (+++) and KDR (+++) (Fig. 1b).

Fluorescence staining

Positive cells were stained red by DiI-ac-LDL and green by FITC-UEA-1; double-positive cells were yellow with DiI-ac-LDL and FITC-UEA-1 (Fig. 1c). Attached cells in all groups were positive for Dilac-LDL as well as FITC-UEA-I staining, and more than 65% adherent cells were double-positive. Highest level of double-positive cells was $92.59 \pm 4.44\%$ in group 40.

Expansion

Numbers of EPCs were compared between groups by counting expansion multiple. Regardless of culture conditions, cells proliferated actively from passage 1 to

Figure 1. Identification of EPCs. (a) After 1 day of culture, primary passage cells were round and non-adherent (1); after 5 days culture, attached spindle-shaped cells (primary passage) increased and formed colonies (2); after 3 days culture, attached spindle-shaped cells (fourth passage) reached near confluence (3). (b) the majority of cells positively expressed CD133, CD34, and KDR, positive cells stained brown/reddish brown. (c) Positive cells stained red with DiI-ac-LDL and green with FITC-UEA-1; double-positive cells were stained yellow by DiI-ac-LDL and FITC-UEA-1.

passage 4 and reached a peak after 3 days culture. The expansion multiple (131.22 ± 6.5) was highest when culture density was $1 \times 10^4/\text{cm}^2$, and culture medium was M199 supplemented with 10% FBS and VEGF + bFGF + EGF + IGF (Table 2).

Migration

Migratory capacity of every group of cells increased in varying degrees through the *in vitro* culture. It increased obviously when stimulated by optimal culture conditions (group 40), when migration level ($20.08 \pm 0.93\%$) of group 40 cells was maximal in all groups (Table 2).

Result of orthogonal group

Results showed that the four factors, culture density (a), basic medium (b), FBS concentration (c) and cytokines (d), were significant related to expansion multiple from passage 1 to passage 4 and migration level (Table 3). In terms of expansion multiple and migration level, degree of its effect was $b > d > a > c$, $d > b > a > c$; b was highly related to c, and c was highly related to d. Optimal culture conditions were $1 \times 10^4/\text{cm}^2$ (a), M199 (b), 10% FBS (c) and VEGF + bFGF + EGF + IGF (d).

Table 3. Results of analysis of variance for orthogonal design

Sources of variation	Sum of squares of mean deviation	Degree of freedom	Variance	F	P
Expansion passage 1–4					
a	5820.00428	3	1940.00143	24.47	<0.0001
b	20034.66047	3	6678.22016	84.23	<0.0001
c	1682.98329	3	560.99443	7.08	0.0006
d	14750.05793	3	4916.68598	62.01	<0.0001
b*c	2914.33003	9	323.81445	4.08	0.0008
Error	3330.07075	42	79.28740		
Migration rate					
a	153.5662422	3	51.1887474	1425.46	<0.0001
b	171.5024297	3	57.1674766	1591.95	<0.0001
c	8.5273297	3	2.8424432	79.15	<0.0001
d	225.1680297	3	75.0560099	2090.10	<0.0001
c*d	4.2520357	9	0.4724484	13.16	<0.0001
Error	1.5082315	42	0.0359103		

a, culture density; b, basic medium; c, FBS concentration; d, cytokines.

Discussion

Great advances in research have been made in animal models for ischaemic cardiovascular disease; however at present, studies on therapeutic application of EPCs is

restricted because the method for EPCs culture is not unified and its efficiency is very low (19,20). Thus this study (to the best of our knowledge), for the first time has indicated that number and function of porcine EPCs could be increased by optimized culture *in vitro*. This optimization of culture conditions will be useful in overcoming the problem of limited numbers of EPCs available. However, cells isolated by density-gradient centrifugation from porcine bone marrow were not EPCs but MNCs, that could differentiate into EPCs in standard medium with high concentration of cytokines and FBS, during the first 7 days of *ex vivo* culture (16,21–23). We have attempted to use different media to culture the cells directly after isolation, but such cells showed morphological and phenotypic characteristics different from those of EPCs. Therefore, we used standard medium during the first 7 days of *ex vivo* culture to exclude variation in results caused by differentiation in other media. Although to date, the method for identification of EPCs is not unique, according to the majority of studies (11,16,24,25), our cultured EPCs were confirmed by cell morphology, CD34⁺, CD133⁺ and KDR⁺ expression detected by immunohistochemistry, and Dil-ac-LDL and FITC-UEA-I reaction determined by fluorescence staining.

Recent findings (6,26) have suggested that culture conditions such as culture density, basic medium, serum concentration and cytokine presence, might be correlated to number and function of EPCs. Accordingly, emerging evidence has indicated (8,24,25,27) that such difference is confirmed in choice of cell culture density and culture medium. Density of culture is vital to population growth of cells and proliferation depends on cell–cell interactions. When cells are cultured at low density, it is difficult to induce differentiation and proliferation, and these cells may even rapidly degrade due to lack of support. When cells are cultured at high density, it restrains population growth and cells degrade earlier because of lack of sufficient nutrition; such attempts also waste limited resources. Thus, appropriate cell density promotes cell population growth and does not consume nutrients excessively. We compared four different culture densities (5×10^3 , 1×10^4 , 2×10^4 and $5 \times 10^4/\text{cm}^2$) and found that the optimal culture density was $1 \times 10^4/\text{cm}^2$.

Our investigations have confirmed that composition of culture medium was a main factor, which may affect number and function of EPCs. If culture medium is not suitable, it does not induce growth of EPCs. It has been reported (25,28,29) that appropriate culture media are not unique for EPC culture. Eggermann *et al.* (28) used M199 + 20% FBS as an EPC culture medium, and cells proliferated actively. Tousoulis *et al.* (30) concluded that DMEM + 10% FBS was a well-suited culture medium. Based on recent studies (28–31), we chose four common

basic media (M199, EGM-2, DMEM and 1640) and found that M199 was the best. In addition, blood serum is an indispensable component of culture medium for EPCs. In particular, FBS is commonly used in EPC culture, and concentration of FBS is also an important factor. If serum concentration in the medium is low, cell population growth is restrained, and resultant number of cells is reduced. However, if blood serum concentration higher, it can neither induce cell proliferation, nor decrease cost of culture. Recently, numerous studies (28,30,32) have suggested that general FBS concentration of 20% or 10%, and a few reports (28,33,34) have concluded that common usage was 5% or 2% FBS. We compared four different concentrations of FBS (2%, 5%, 10% or 20%) and demonstrated that optimal concentration was 10% in our *in vitro* culture of EPCs; this level could both increase number of EPCs and decrease cost of the cultures. Present findings have confirmed that there was a significant relationship between basic medium and FBS.

Cytokines also play an important role in promotion of cell population growth. If only a single cytokine was used or necessary cytokines were missed, cell proliferation slowed down, and even cell death occurred. If combinations of cytokines were used, proliferation speeds up. Numerous findings (14,35) have suggested medium supplemented with VEGF only but Casamassimi *et al.* (36) used VEGF + bFGF + EGF in EPC culture medium; however, growing numbers of reports (35–38) have confirmed that VEGF is necessary and is the most important cytokine for EPC culture. Although no report has shown that bFGF, IGF, or EGF has been used separately in cultured EPC medium, main findings of (31,33,39) have suggested that roles for bFGF, IGF, or EGF were certain, and that their combination was better than alone. Thus, we studied four most commonly used combinations of cytokine (VEGF, VEGF + bFGF, VEGF + bFGF + EGF and VEGF + bFGF + EGF + IGF) and demonstrated that the optimal combination was VEGF + bFGF + EGF + IGF. In the light of numerous data (25,34,38,39), we chose our culture medium to be 10 ng/ml of VEGF, 50 ng/ml of bFGF, 20 ng/ml of EGF and 50 ng/ml of IGF.

It is possible that our results may have missed some information on combination of other culture conditions. For example, another interesting cytokine with well-established interactions with endothelial progenitor cells is stromal derived factor-1 (SDF). However, we chose the four main factors and compared four most commonly used levels for EPC culture. In addition, combinations of other factors and levels were less effective in EPC culture. Meanwhile, we used orthogonal design to reduce number of groups and optimize culture conditions. Therefore, we believe that results of this study were reliable and the findings were valid.

To summarize, we have confirmed that number and migratory capacity of EPCs increased in relation to cultured cell density, basic medium, FBS concentration and cytokines. Thus, optimal culture conditions of EPCs from porcine bone marrow were as follows: culture density was $1 \times 10^4/\text{cm}^2$ and culture medium was M199 supplemented with 10% FBS and VEGF + bFGF + EGF + IGF. These findings imply that we established an optimal culture method to lay the foundation for transplanting EPCs to treat ischaemic diseases. However, the culture method of EPCs has not been standardized totally; future studies for optimization of culture conditions, such as culture density of MNCs and concentration of cytokines should be carried out to obtain EPCs with high efficiency and functional quality.

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