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Culture conditions of human embryonic stem cells for differentiation into retinal vascular structure

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Abstract. – OBJECTIVE: This study sought to identify the suitable cell culture conditions for the in vitro-induced differentiation of human embryonic stem cells (hESCs) into retinal vascular tissue cell types.

MATERIALS AND METHODS: To do this, we established four treatment groups. Group A was designed to culture hESCs in a three-dimensional system. The feeder cells and leukemia inhibitory factor (LIF) were removed in Group B. In group C, hESCs were cultured with a variety of pro-angiogenic growth factors. In group D, hESCs were cultured with intact retinal support cells and extracellular matrix. On days 15 and 30, the expression of platelet endothelial cell adhesion molecule 1 (PECAM1), α-smooth muscle actin (αSMA), and macrophage marker F4/80 were detected by immunofluorescence staining. ELISA was used to detect the expression of stromal cell-derived factor-1 (SDF-1).

RESULTS: At both 15 and 30 day timepoints, the highest PECAM1, αSMA, and F4/80 positive rates and SDF-1 expression levels were observed in group D, followed by group C, group B, with group A presenting the lowest expression of these proteins (*p*<0.05). Also, group D showed obvious angiogenesis structures.

CONCLUSIONS: Our study indicates that hESCs can differentiate into retinal vascular-like structures. The presence of intact retinal support cells, a variety of cytokines, and extracellular matrix components were essential to facilitate this differentiation.

Key Words:

Human embryonic stem cells, Retinal neovascularization, Platelet endothelial cell adhesion molecule 1, Đ-smooth muscle actin, Macrophage marker F4/80, Stromal cell derived factor-1.

Introduction

Retinal neovascularization can disrupt the normal chorioretinal structure, impacting the generation and conduction of visual signals. In severe cases, retinal neovascularization could lead to retinal bleeding and fluid leakage. Retinal neovascularization has been linked to the incidence of more than 40 blinding eye diseases[1]. Its development involves ischemia, hypoxia, and inflammation. A variety of effector cells and cytokines have been shown to participate in these processes[2]. Studies with animal models have found[3] that neovascularization occurs due to both on-site proliferation of tissue cells and contributions of bone marrow-derived cells (BMCs), suggesting the possibility of stem cell therapy. Simple application of vascular endothelial growth factor (VEGF) antagonist or antibody could only partially reverse disease progression, and a high recurrence rate was maintained, indicating that VEGF was only one of the important factors in neovascularization[4]. Human embryonic stem cells (hESCs) are pluripotent stem cells capable of self-renewal and differentiation into multiple germ layers, but the conditions to induce desired differentiation remain a "black box" to be studied. Exploring the conditions of differentiation is of great importance for research and disease treatment. Moon et al[5] implanted Matrigel encapsulated multicellular hESC-derived endothelial cells in an ischemic disease model and found that implantation could repair damaged tissues and promote regeneration without side effects such as tumorigenesis. Lu et al[6] treated retinal diseases caused by ischemic-reperfusion injury or diabetes with intravenous or intravitreal injections of hESCs in mice. About 30 to 60% of the hESC-derived endothelial cells were involved in retinal neovascularization and maturation, and they were found to be distributed in multiple retinal cell layers. Based on these previous researches, our study investigated the suitable cell culture conditions for *in vitro* induced differentiation of hESCs into retinal vascular structures.

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Materials and Methods

Materials

Both hESCs and retinal support cells were obtained from the cell center of the Hubei University of Medicine. The feeder cell line SIM Thioguanine/Ouabain-resistant mouse fibroblast (STO) (ATCC, CRL1503™) was purchased from Sangon Biotech (Shanghai, China). Fetal bovine serum (FBS), glutamine, and trypsin were purchased from Beyotime Biotechonology (Haimen, Jiangsu, China). Non-essential amino acids (NAAs), mitomycin C (MMC), gelatin, and type I collagen were purchased from Zhongshan Golden Bridge Biotechnology Co. (Beijing, China). VEGF, basic fibroblast growth factor (bFGF), and interleukin -6 (IL-6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM) and recombinant human leukemia inhibitory factor (LIF) were purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-human platelet endothelial cell adhesion molecule 1 (PECAM1), α-smooth muscle actin (αSMA), and macrophage marker F4/80 monoclonal antibodies were purchased from Bio-Rad (Hercules, CA, USA). TRITC labeled goat anti-mouse IgG secondary antibody was purchased from R&D (Minneapolis, MN, USA). Anti-fluorescent quencher was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stromal cell derived factor-1 (SDF-1) kit was purchased from Media Cybernetics (Rockville, MD, USA). The study was approved by the Ethics Committee of Xiangyang No. 1 People's Hospital. Micropipettes, Eppendorf tubes, and Millicell hanging cell culture chambers were purchased from GE (Fairfield, CT, USA). CO_2 incubator, refrigerators, and a Elix/RiOs water purification system were purchased from Millipore Corporation (Billerica, MA, USA). A fluorescence microscope was purchased from Olympus (Tokyo, Japan). The paraffin-embedding system and microtome were purchased from Leica (Wetzlar, Germany).

Cell Culture

The hESCs and retinal support cell lines were thawed and subcultured until cell volume was greater than 85% confluence. After trypsin digestion and subsequent termination, cells were resuspended in phosphate buffered saline (PBS) at a concentration of 2×10^7/ml. STO cells were seeded in 0.1% gelatin-coated 100 mm tissue culture dishes and incubated at 37°C with 5% CO_2. After cells formed confluent monolayers, they were cultured for another 2 days. MMC was used at a final concentration of 10 μg/ml to treat cells for 2 h. After trypsin digestion, separated STO cells were added to the medium for hESCs (high glucose DMEM + 15% fetal calf serum (FCS) + 1% NAAs + 0.1 mol/Lβ- mercaptoethanol + 2 mol/L glutamine + 100 U/ml penicillin + 100 μg/ml streptomycin + 10 ng/ml recombinant human LIF). STO cells were inoculated into gelatin-coated tissue culture dishes at a ratio of 1:5 (STO cell to hESCs medium) and cultured in suspension. The medium was changed every day.

Experimental Protocols

There were four treatment groups used in this study. In group A, hESC cells were cultured in a three-dimensional system. In group B, feeder cells and LIF were removed from the medium. In group C, hESCs were cultured with a variety of pro-angiogenic growth factors. In group D, hESCs were co-cultured with retinal support cells and extracellular matrix. The culture conditions for group A were previously described above. Embryoid bodies (EBs) were prepared in advance for group B. Briefly, hESC suspensions were inoculated in gelatin-coated tissue culture dishes and incubated at 37°C with 5% CO_2 for 3 h. At this time point, most of the STO cells were adherent while the hESCs were still suspended in the supernatant. The supernatant was then collected and centrifuged, and the pellet was resuspended in EB culture medium and inoculated into tissue culture dishes for suspension culture. For group C, EBs were cultured with 50 ng/ml VEGF, 100 ng/ml bFGF and 10 ng/ml IL-6. This culture was performed for 10 days without changing the media. On day 11, EBs were collected by centrifugation and precipitation. Cells were then washed with PBS. 1.2 ml medium (180 μl/ml 5 × DMEM + 330 μl/ml 3 × A medium + 10 μl/ml 100 × growth factor mixture + 100 μl/ml 1 × DMEM + 380 μl/ml H_2O; 1 × A medium: 15% fetal calf serum (FCS) + 1% penicillin-streptomycin +1% β- mercaptoethanol +1% NAAs) was prepared on ice. Fifty EBs were resuspended in the above medium rapidly and inoculated in 30 mm tissue culture dishes. The dishes were incubated at 37°C with 5% CO_2 and fixed for 30 min. hESCs in group D were co-cultured with retinal support cells and 1 mg/ml type I collagen.

Monitored Indicators

On days 15 and 30, the expression of PECAM1, αSMA, and macrophage marker F4/80 were monitored by immunofluorescence staining. The expression of SDF-1 was detected by ELISA. For immunofluorescence staining, EB cells were fixed with paraformaldehyde and dehydrated by sucrose overnight at room temperature. Then, cells were embedded in paraffin. Frozen slices (thickness 5 μm) were dewaxed and underwent antigen retrieval, endogenous peroxidase blocking, and incubations with primary and secondary antibodies. Slices were then observed under a microscope. Ten slices were randomly selected for each group. Five vision fields (up, down, left, right, and central) were observed for each slide. The percentages of positive staining cells were calculated and the average rate was reported. Three-dimensional vascular structures were scanned by a fluorescence microscope from top to bottom in 0.1 μm layer increments. 500 images were collected and analyzed using Flow-view 4.3 software.

Statistical Analysis

Statistical analyses were conducted using SPSS20.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed as mean ± standard deviation. Among-group comparisons were performed using one-way ANOVA. Pairwise comparisons were performed using LSD-t test. Intra-group comparisons (30 d *vs.* 15 d) were performed using paired *t*-test. $p<0.05$ was considered statistically significant

Results

Immunofluorescence Staining Results

On days 15 and 30, group D had the highest PECAM1, αSMA, and F4/80 positive rates, followed by group C and group B, while group A showed the lowest rates ($p<0.05$, Table I).

Analysis of Angiogenesis

The hESC cells in group A remained undifferentiated in the presence of the feeder layer and LIF. Cells appeared in round or oval colonies with clear and regular edges. Inside the colonies, cells were tightly packed with no obvious boundaries. The hESC cells in group B differentiated into a single-layer primitive endoderm, which was next to the basement membrane. A primitive ectoderm with polarized arrangements appeared adjacent to the basement membrane, but no endothelial cell (EC) network formed. ECs and dense EC networks could be seen in group C. In group D, cord-like, tube-like, and irregular cell clusters could be observed. EBs were rich in EC networks and tube-like structures.

SDF-1 Expression

On days 15 and 30, group D had the highest SDF-1 expression level, followed by group C and group B, while group A showed the lowest expression of SDF-1 ($p<0.05$, Figure 1).

Discussion

The generation of new blood vessels includes neovascularization and angiogenesis. Neovascularization refers to the process in which angioblast cells differentiate into ECs and directly form an original vascular network, while angiogenesis is the formation of new blood vessels and networks from EC budding out from existing vascular networks. Both neovascularization and angiogenesis are involved in the formation of new retinal vessels, although angiogenesis is more prominent. Retinal local or circulating stem cells

Table I. Immunofluorescence staining results (% positive cells).

	PECAM1		αSMA		F4/80	
Group	**15 d**	**30 d**	**15 d**	**30 d**	**15 d**	**30 d**
A	3.5±0.9	4.2±1.1	2.6±0.6	2.9±0.7	5.2±2.3	5.5±2.2
B	12.3±4.6	16.8±7.5	8.9±2.2	11.4±3.6	15.8±5.5	21.2±8.9
C	22.6±9.3	32.4±12.2	16.7±7.8	23.5±10.2	29.8±11.4	45.2±25.3
D	35.6±12.3	59.7±25.6	26.9±11.5	36.7±13.6	46.9±25.5	67.5±32.5
F	16.528	24.256	20.215	32.625	54.628	72.635
p	0.000	0.000	0.000	0.000	0.000	0.000

$^{\#}p < 0.01$; $^{*}p < 0.05$

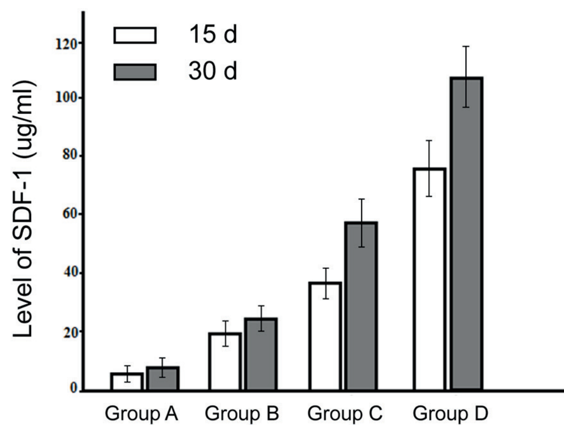

Figure 1. Expression of SDF-1. Group C and D had significantly increased SDF-1 expression on day 30 compared with day 15, while groups A and B did not show significant change between the two timepoints. Group D had the highest level of SDF-1 on day 15 and 30, followed by groups B and C. Group A showed the lowest SDF-1 expression.

aggregated at the lesion sites through chemotaxis. Retinal support cells, cytokines, and extracellular matrix in the local microenvironment induce the formation of new retinal vessels[7,8]. This process formed the theoretical basis of this work. Our study showed that after removing feeder cells and LIF, hESCs could differentiate into a primitive blood vessel endoderm structure. Pro-angiogenic growth factor induction could facilitate hESC differentiation into EC networks, where increased expression of PECAM1 (a marker for endothelial cells), αSMA (smooth muscle marker), and macrophage marker F4/80 were observed, as well as the secretion of the extracellular matrix component SDF-1, suggesting that the observed cells functioned like vascular tissues. In the presence of intact retinal support cells and extracellular matrix, hESCs differentiated into more mature vascular structures, and the proportion of cells derived from hESCs increased significantly. Microenvironments can regulate the cell cycle and differentiation of stem cells[9]. Smooth muscle structure plays an important role in the secretion of growth factors and matrix deposition. The proportion of smooth muscle has been found to be closely related to the degree of maturation of newly formed vessels[10]. Macrophage infiltration is a response to monocyte chemotactic protein produced by the retinal pigment epithelium. These cells are able to secrete VEGF and tissue factor (TF), and TF could promote cellulose production, providing growth scaffolds for new vessels[11,12]. Sakurai et al[13] noted that macrophages were a key factor in the initiation of laser-induced retinal neovascularization. Also, SDF-1 could mediate endothelial selectin (E-selectin) expression, which could attract stem cells to appropriate target sites through chemotaxis. VEGF could also induce the expression of E-selectin, thereby enhancing the ability of SDF-1 to recruit circulating stem cells to the choroid[14,15]. In addition to embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells, endothelial progenitor cells, and multipotent adult stem cells have also been widely applied in animal models to study retinal neovascularization[16,17]. Interventions to reduce stem cell aggregations in the retina may be an effective method of treating retinal neovascular diseases[18,19]. Currently, the key factors directing stem cell differentiation, the interactions between stem cells and their microenvironment, as well as the critical cell components in differentiation, remain unclear. In addition, the journey from bench to bedside is long. Nonetheless, this study showed that hESCs can differentiate into retinal vascular-like structures, while intact retinal support cells, a variety of cytokines, and extracellular matrix were necessary for the differentiation. This research provides a solid framework for further explorations.

Conclusions

We showed that hESCs can differentiate into retinal vascular-like structures. The presence of intact retinal support cells, a variety of cytokines, and extracellular matrix components, were essential to facilitate this differentiation.

Conflict of interest

The authors declare no conflicts of interest.

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