Nestin expression during differentiation of fetal endothelial progenitor cells, and hypoxic culture of human umbilical vein endothelial cells

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<ABSTRACT>

Nestin expression during differentiation of fetal endothelial progenitor cells, and hypoxic culture of human umbilical vein endothelial cells

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Objective: Recent reports have documented that Nestin, a type VI intermediate filament protein could be used as a marker of angiogenesis and neovascularization capacity of endothelial cell. Our previous study showed that the Nestin expression was different in placentas from normal pregnant women and women with severe preeclampsia. Umbilical cord blood EPCs play an important role in angiogenesis, organogenesis, and vascular repair by the processes. The aim of this study is to assess whether Nestin is expressed during differentiation of EPCs obtained from human umbilical cord blood (HUCB), and its expression is changed in hypoxia-conditioned culture of human

umbilical vein endothelial cells (HUVECs).

Materials and Methods: Among deliveries at our institute, 15 normal pregnant women who delivered by cesarean section at 37 ~ 40 weeks of gestation were selected. About 50 ml of umbilical cord blood and cord at the time of delivery were obtained. Human umbilical cord blood mononuclear cells (MNCs) from HUCB were isolated and cultivated on 6-well plates coated with human fibronectin in endothelial basal medium-2 (EBM-2). Cultures were maintained for day 7. On the seventh day, EPCs were characterized by detecting the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (acLDL-DiI), and FITC-labeled Ulex europaeus agglutinin-1 (UEA-1). After the characterization of EPCs, cell culture was continued through day 15 for differentiation to outgrowth endothelial cell (OEC). HUVECs were isolated from human umbilical cord veins by collagenase treatment, and were grown in M199 medium. For normoxia experiments, the HUVECs were cultured at 37 ℃ in humidified 5% CO_2/95% air, and for hypoxia, they were cultured at 37 ℃ with 5% CO_2, 94% N_2, and 1% O_2 in a multi-gas incubator. Culture in hypoxic conditions was performed for 24 hours. Nestin gene expression in EPCs, OECs, and HUVECs was detected by semi-quantitative RT-PCR (sqRT-PCR). To identify hypoxic effects in culture of HUVECs, VEGF expression was co-detected.

Results: sqRT-PCR revealed that Nestin gene was downregulated in EPC, but upregulated in OECs and HUVECs. During 24 hours of HUVEC culture, time

course gene expression of VEGF was significantly increased, but Nestin was not changed.

Conclusions: These results suggested that Nestin could be used as a new differentiation marker of fetal endothelial cell. But our studies showed that hypoxic stimuli did not directly affect nestin gene expression.

Key words : Nestin, Endothelial progenitor cell, Human umbilical vein endothelial cell

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I. INTRODUCTION

Nestin is a type VI intermediate filament protein originally described in neural stem cells.[1,2] In undifferentiated cells, nestin expression is transient during cell differentiation. But after differentiation, nestin expression is down-regulated and apparently replaced by another type of intermediate filament, such as glial fibrillary acidic protein (GFAP), neurofilaments and desmin.[3,4] Recent reports have documented nestin expression in the endothelium of blood vessels supplying the intact central nervous system,[5] tumors,[6] and the

limb bud of developing mouse.[7] Its expression is shown to be confined to newly formed endothelial cells.[8] In human tissue, nestin positive endothelial cells are found in vessels of the corpus luteum associated with neovascularization, of regenerating myocardium and of the core of chorionic villi in the human placenta.[8] Furthermore, Shimizu T et al. showed that nestin could be used as a marker of angiogenesis and neovascularization capacity of endothelial cell and suggested that it might be superior to proliferating cell nuclear antigen (PCNA) as a marker for growing cells.[9] Our previous study showed that the nestin expression was different in placentas from normal pregnant women and women with severe preeclampsia.[10] And we hypothesized that increased nestin in pre-eclamptic placenta may be due to the following: first, the uteroplacental hypoxia induced by pre-eclamptic conditions may have increased nestin gene expression, second, endothelial progenitor cells with relatively high angiogenic activity could have homed to placental endothelium in greater amounts and this may give an explanation for increased nestin expression.

Endothelial progenitor cells (EPCs) were first isolated from peripheral circulating mononuclear cells by Asahara et al. in 1997.[11] Accumulating evidences that EPCs play an important role in endothelium maintenance have been implicated both in reendothelialization and in neovascularization.[12-16] Umbilical cord blood EPCs play an important role in angiogenesis, organogenesis, and vascular repair by the processes.[17,18]

Until now, there have been no report on nestin expression in EPC and mature

endothelial cell (outgrowing endothelial cell, OEC) differentiated from it, and change of nestin expression in endothelial cell affected by hypoxic insult. The aim of this study is to assess whether nestin is expressed during differentiation of endothelial progenitor cells obtained from human umbilical cord blood and hypoxia-conditioned culture of human umbilical vein endothelial cell.

II. MATERIALS AND METHODS

1. Study population and sample collection

Among deliveries at our institute, only those who delivered by cesarean section at 37-40 weeks of gestation were selected. About 50 ml of umbilical cord blood were obtained at the time of delivery. Pregnancies associated with premature rupture of membranes, fetal malformations, chromosome anomalies, multiple pregnancies, small for gestational age, preeclampsia, hypertension, renal or endocrine diseases were excluded from the study.

According to the inclusion and exclusion criteria, 15 patients were eligible to be included, and their data were analyzed. All patients gave consent to the sampling and use of their medical records for future research. At the time of delivery, cord was clamped after the fetal expulsion and subsequently HUCB for EPC isolation and HUVEC were obtained.

2. Isolation and cultivation of EPC and OEC

We isolated human umbilical cord blood mononuclear cells from HUCB. HUCB samples (about 50 ml each) were collected from fresh placentas with attached umbilical cords by gravity flow. MNC were isolated by density gradient centrifugation over Biocoll (Biochrom, Berlin, Germany) for 30 min at 400×g and washed three times in PBS (Biochrom) and seeded 107 cells on 6-well plates coated with human fibronectin (Sigma-Aldrich Chemie, Munich, Germany) in endothelial basal medium-2 (EBM-2) (Clonetics, Cell Systems, St

Katharinen, Germany). The medium was supplemented with endothelial growth medium 2 (EGM-2) Single Quots (Clonetics, Cell Systems) containing fetal bovine serum, human vascular endothelial growth factor A (VEGF-A), human fibroblast growth factor-B, human epidermal growth factor, insulin like growth factor-1, and ascorbic acid in appropriate amounts. After 3 days non-adherent cells were removed and fresh culture medium was applied. Cultures were maintained through day 7. Phenotypical analysis of the cells was performed on days 3, 5 and 7. After characterization of EPCs at day 7, culture for each cell was continued through day 15 for differentiation to OEC.

3. Characterization of EPC and OEC

We performed fluorescent chemical detection to determine the cell type of the attached human umbilical cord blood mononuclear cells after 7 days of culture. To detect the uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine –labeled acetylated low-density lipoprotein (acLDL-DiI) (Molecular Probes, Leiden, The Netherlands), we incubated the cells with acLDL-DiI (6 μg/mL) at 37°C for 2 hours. Cells were then fixed with 1% paraformaldehyde for 10 minutes and incubated with FITC-labeled Ulex europaeus agglutinin-1 (UEA-1) (Sigma) for 1 hour. After the staining, we viewed the samples with an inverted fluorescent microscope (Leica, Heidelberg, Germany). For identification of EPC and OEC, RT-PCR was performed for each specific cell markers such as AC133, CD45, CD34, vWF (von Willebrand factor), eNOs (endothelial

constitutive NO synthase), and KDR (kinase-insert domain containing receptor) (BIONEER, Inc. CA, USA). The primers used for AC133, CD45, CD34, vWF, eNOs, and KDR were as follows: AC133 (sense: 5'- acttggctcagactggtaaa -3' and antisense: 5'- gttctgagcaaaatccagag -3'), CD45 (sense: 5'- cctggagaaccctttatttt -3' and antisense: 5'- ttcatcagcaatcttcctct -3'), CD34 (sense: 5'- ctctcacctgtactcttccg -3' and antisense: 5'- cagctggtgataagggttag -3'), vWF (sense: 5'- caaggtcaatgagagaggag -3' and antisense: 5'- gtcaatggagtacatggctt -3'), eNOs (sense: 5'- tggtcaactatttcctgtcc -3' and antisense: 5'- accacgtcatactcatccat -3'), KDR (sense: 5'- ttcaatgtgaagctgtgtgt -3' and antisense: 5'- ttactggagtagaggccaaa -3').

4. Isolation and hypoxic cultivation of HUVEC

HUVECs were isolated from human umbilical cord veins by collagenase treatment as described previously,[19] and used in passages 2-7. The cells were grown in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 3 ng/ml bFGF (Upstate Biotechnology, Lake Placid, NY), and 5 units/ml heparin at 37°C. For normoxia experiments, the HUVECs were cultured at 37°C in humidified 5% CO2/95% air, and for hypoxia experiments, the HUVECs were cultured at 37°C with 5% CO2, 94% N2, and 1% O2 in a multi-gas incubator (Juji Field, Inc., Tokyo, Japan). Culture in hypoxic conditions was performed for 24 hours.

5. Semi-quantitative RT-PCR Analysis

Total RNA was obtained from EPCs, OECs, and HUVECs with a TRIzol reagent kit. 0.5-5 μg RNA samples were used in the RT-PCR, and the correlation between the amounts of RNA used and quantity of PCR products from nestin mRNA and the internal standard (GAPDH) mRNA was examined. Briefly, target RNA was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, and 1 mM dNTPs at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min. One μl of the cDNA mixture was used for enzymatic amplification. The polymerase chain reaction was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 μM of primers for nestin. Amplification was performed in a DNA thermal cycler (model PTC-200; MJ Research) under the following condition: denaturation at 94°C for 5 min for the first cycle and for 30 s thereafter, annealing at 60°C (VCAM-1), for 30 s, and extension at 72°C for 30 s for 25 repetitive cycles. Final extension was at 72°C for 10 min. To evaluate hypoxic effects in culture of HUVEC, VEGF and nestin expressions were co-detected by RT-PCR. The primers of VEGF and nestin used in hypoxic culture were as follows: VEGF (sense: 5'-gtggacatcttccaggagta-3' and antisense: 5'-gcgagtctgtgtttttgc-3'), and nestin (sense: 5'-cgatctcttgacatacggtt-3' and antisense:

5'-aggggacctagtactatcgg-3'). Each experimental condition was performed in quadruplicate.

6. Densitometric analysis

RT-PCR results were visualized with densitometric scanning using the densitometer (IMAGE READER LAS-1000 lite, Fuji Photo Film Co. Ltd. Japan). Densitometry was carried out with digital analysis software (Fuji Photo Film Co. Ltd. Japan).

7. Statistical analysis

Patient's characteristics were expressed as the mean ± SD. Statistical differences for densitometric data in RT-PCR results were tested using Student's t test using SPSS, version 12.0. A *p*-value of less than 0.05 was considered to be statistically significant.

III. RESULTS

1. Clinical characteristics of patients

The clinical characteristics of the patients who provided umbilical cord blood and cords for studies were presented in Table 1. Maternal age, gestational age at delivery, birth weight, parity, and abortion were 30 ± 3.4 years, 38.4 ± 1.6 weeks, 3149 ± 409 g, 1.0 ± 0.9, and 0.8 ± 1.1.

TABLE 1. Clinical characteristics of the patients

Variable	Study group (n = 15)
Maternal age (years)	30 ± 3.4
Gestational age at delivery (weeks)	38.4 ± 1.6
Birth weight (g)	3149 ± 409
Parity	1.0 ± 0.9
Abortion	0.8 ± 1.1

Data: Mean ± SD.

2. Isolation and cultivation of EPC and OEC

Human umbilical cord blood mononuclear cells (MNCs) were isolated from 50 ml human umbilical cord blood. After removal of non-adherent cells, EPCs were collected and cultivated. Cultures were maintained through day 10. In early stage, EPCs were small size and oval shape. After differentiation, OECs (day 10) were identified by its specific spindle shape. Both EPCs and OECs were detected in plates from day 5 to day 7 (Figure 1).

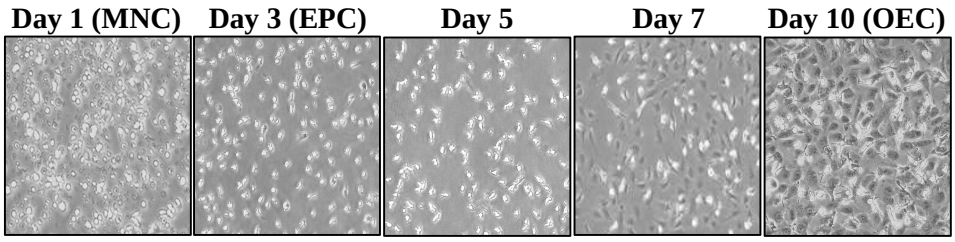


Figure 1. Isolation and cultivation of EPCs and OECs formation. Human umbilical cord blood mononuclear cells (MNCs) were isolated from human umbilical cord blood. MNCs were cultivated on plate coated with human fibronectin. After 3 days non-adherent cells were removed and fresh culture medium was applied. Cultures were maintained through day 10. Phenotypical analysis of the cells was performed on day 3, 5, 7 and 10.

3. Characterization of EPC and OEC

Fetal EPCs were stained doubly by both 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (acLDL-DiI) and FITC-labeled Ulex europaeus agglutinin-1 (UEA-1) (Fig. 2A). They were positive for cell markers such as AC133, CD45 and CD34 but did not express vWF, eNOs, and KDR by RT-PCR (Fig. 2B). After identification by fluorescent staining, EPCs were differentiated into OECs. OECs were identified by its characteristic shape. For confirmation of OECs, cell marker identification was performed by RT-PCR. OECs were positive for the expression of vWF, eNOs, CD34, and KDR, but negative for AC133 and CD45 (Fig. 2B). Among the cell markers, CD34 expression was proportionally

increased according to degree of differentiation.

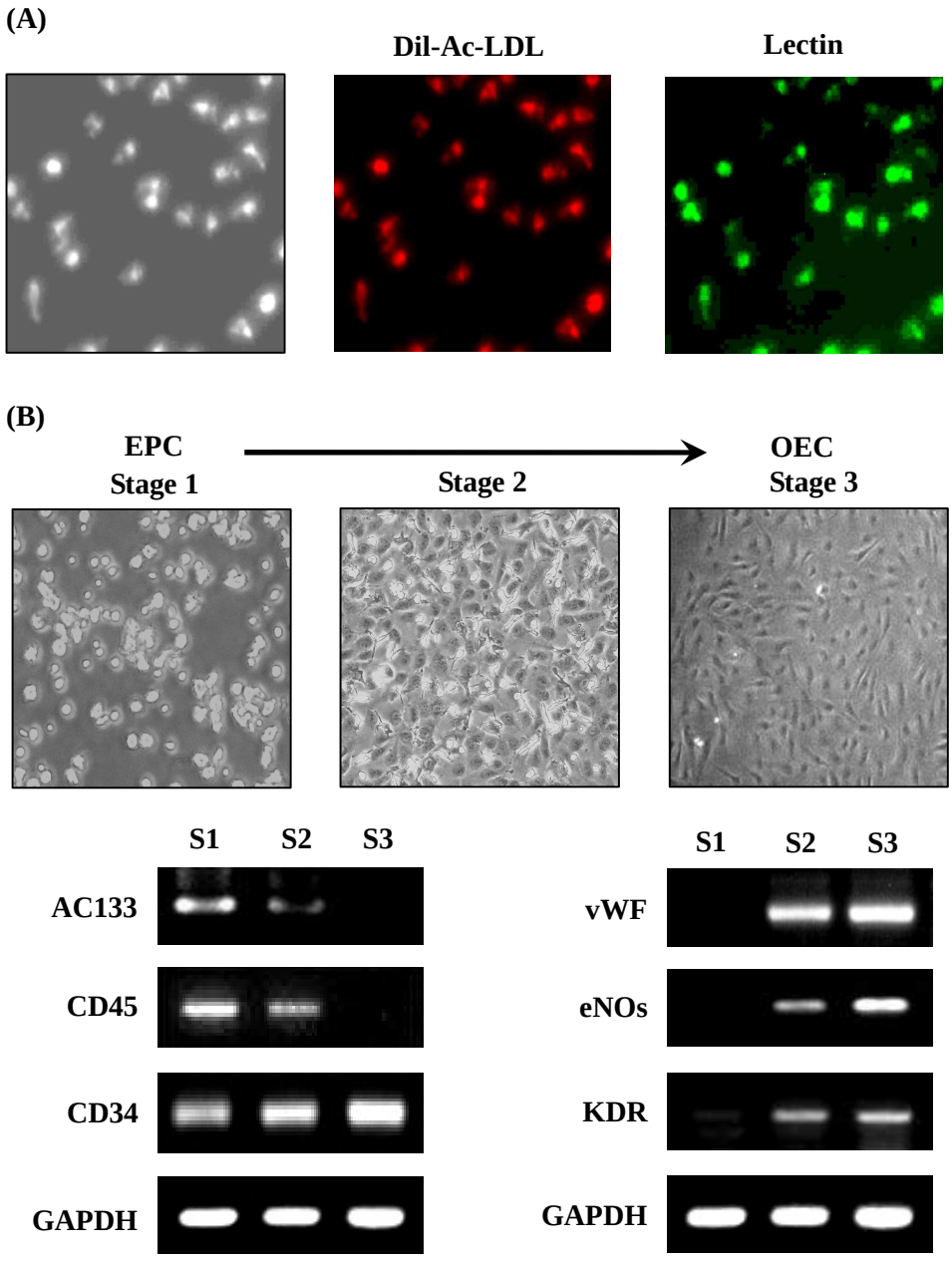


Figure 2. Characterization of EPC and OEC. A. EPCs were stained doubly by both 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated

low-density lipoprotein (acLDL-DiI) and FITC-labeled Ulex europaeus agglutinin-1 (UEA-1). B. EPC and OEC were identified by its characteristic shape and specific cell markers. EPCs (Stage 1) were differentiated into OECs (Stage 3). EPCs were positive for cell markers such as AC133, CD45, and CD34 but did not express vWF, eNOs, and KDR by RT-PCR. OECs were positive for the expression of vWF, eNOs, CD34, and KDR, but negative for AC133 and CD45. S1: Stage 1, S2: Stage 2, S3: Stage3.

4. Nestin gene expression by RT-PCR in EPC, OEC, and HUVEC

RT-PCR was performed in confirmed EPC and OEC, and HUVEC isolated from human umbilical cord (Figure3). Nestin gene was not expressed in EPC but was activated in OEC differentiated from EPC. HUVEC obtained from cord expressed nestin gene like OEC.

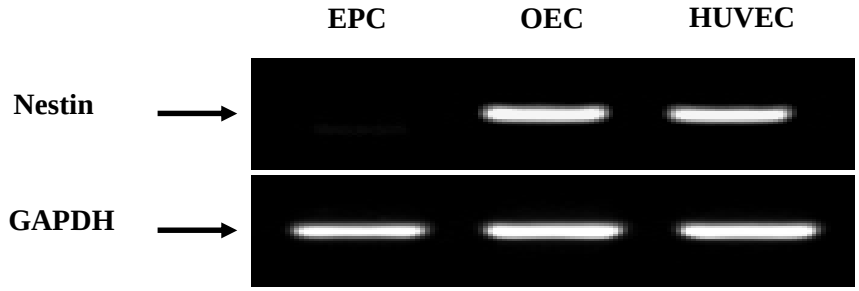
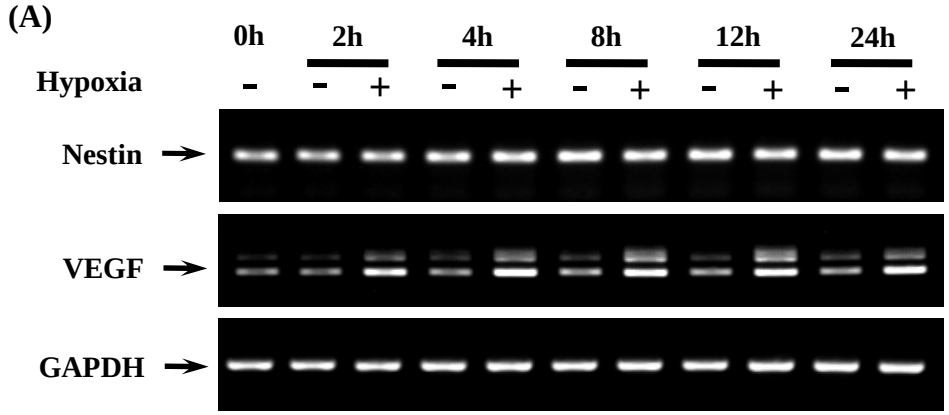
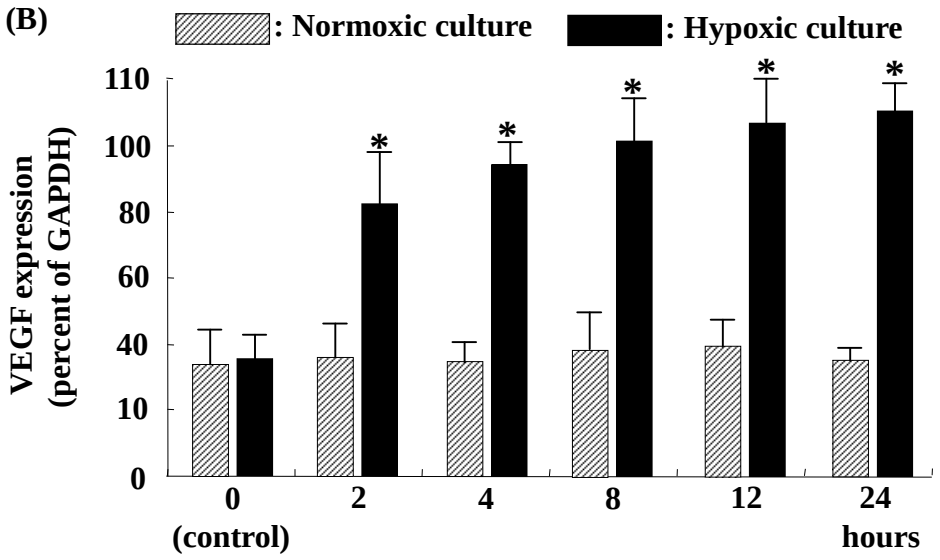


Figure 3. Nestin gene expression by RT- PCR in EPC, OEC, and HUVEC. The expression of nestin gene in EPC, OEC, and HUVEC was assessed using RT-PCR. Nestin expression was observed in OEC and HUVEC. There was no expression of nestin in EPC.

5. Nestin gene expression in normoxic and hypoxic culture of HUVEC

Both VEGF and nestin were normally expressed in control HUVEC (0 h). Nestin and VEGF gene expression were detected every 2 hours during HUVEC culture by RT-PCR. Time course gene expression of VEGF and nestin in HUVEC culture was examined in hypoxic and normoxic condition from 0 h (control) to 24 h (Figure 4A). The VEGF expression was not changed during normoxic culture but increase in hypoxic culture (Figure 4B). In 2 h of hypoxic culture, VEGF expression was sharply increased. From 2 h to 24 h, gentle increase was noted in VEGF expression. In nestin gene expression, there was no change in both normoxic and hypoxic culture conditions (Figure 4C).


(A)
0h
2h
4h
8h
12h
24h
Hypoxia
- - + - + - + - + - +
Nestin
VEGF
GAPDH


(B)
: Normoxic culture
: Hypoxic culture
VEGF expression
(percent of GAPDH)
110
100
80
60
40
10
0
*
0
(control)
2
4
8
12
24
hours

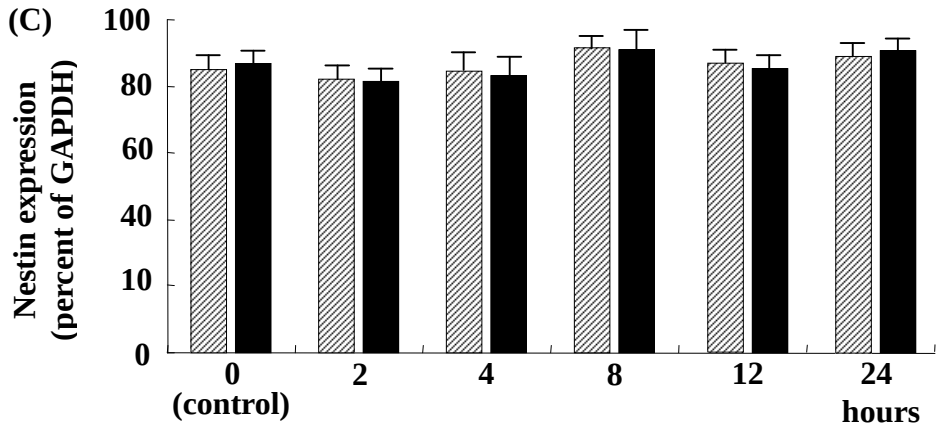

(C)
Nestin expression
(percent of GAPDH)
100
80
60
40
10
0
0
(control)
2
4
8
12
24
hours

Figure 4. Nestin gene expression in normoxic and hypoxic culture of HUVEC. A. Time course gene expression of VEGF and nestin in HUVEC culture was examined by RT-PCR from 0h (control) to 24h. B. The VEGF expression was not changed in normoxic culture but continued to increase at 24 h in hypoxic culture. C. In nestin gene expression, there was no change in both normoxic and hypoxic culture condition. + : hypoxic culture, - : normoxic culture. ***** $p < 0.001$ by Student's *t*-test.

IV. DISCUSSION

The results of this study showed that nestin was expressed in OEC and HUVEC, but not in EPC. Nestin gene expression was not changed in hypoxic culture of HUVEC, which was confirmed by the increase of VEGF expression.

EPCs from bone marrow or peripheral blood play an important role in various physiological and pathophysiological processes. Endothelial damage ultimately represents a balance between the magnitude of injury and the capacity for repair. Although little is known about the mechanisms by which the vessel wall undergoes repair, circulating EPCs have known to play an important role in vascular homeostasis, and constitute one aspect of this repair process. They participate in angiogenesis and arteriogenesis,[20] thus being functionally important in vascular repair. EPCs are also known to play a critical role in angiogenesis of tumor or diseases involving endothelial dysfunction.[21] Promising therapeutic strategies are based on the concept of EPCs being differentiated into mature endothelial cells (OEC). These cells may contribute to vascular repair processes and are expected to be of use for targeted antiangiogenic therapy of malignant tumours.[21] Studies have suggested that an impairment in the number and function of EPCs is observed in diseases related to endothelial dysfunction. For future therapeutic use of putative EPCs further insight into their differentiation, marker profile and potential physiological role is needed. Accumulating studies suggested that AC133, CD45, CD34, vWF, eNOs, and KDR could be used as differentiation markers of EPC.[22-24] Thus,

these markers were used in this study. But, no uniform definition of EPC has been proposed. From results of this study, we suggest that nestin could be used as new differentiation marker of EPC.

Nestin could be used as a marker for proliferation and neovascularization capacity of endothelial cell. Sugawara et al. showed that nestin could be used as a marker for proliferative endothelium in gliomas.[25] Lardon et al. demonstrated that nestin was expressed in angiogenic endothelial cells.[26] Morky et al. reported that cerebral angiogenesis showed nestin expression in endothelial cell, and that angiogenesis of extra- and intraembryonic blood vessels was associated with expression of nestin in endothelial cells.[27,28] Our previous study showed that the expression of nestin was significantly higher in preeclamptic patients compared with normal pregnancies.[10] It was suggested that hypoxic insult from preeclamptic condition may directly affect nestin gene expression. But, in vitro, our studies showed that hypoxic insult did not directly stimulate nestin gene expression.

Expression of stromal cell-derived factor-1, which is important for recruitment of EPCs,[29] is stimulated by tissue hypoxia through the action of hypoxia-inducible factor-1.[30] Thus, it is possible that increased recruitment of EPCs with relatively high expression of nestin explain higher expression of nestin in preeclamptic placenta compared to normal. In next study, it will be considered whether EPC with high expression of nestin has more increased homing to placental endothelium in preeclampsia. Accumulating studies have

suggested that an impairment in the number and function of EPCs is observed in diseases related to endothelial dysfunction. It has been known that damaged EPCs have poor ability of differentiation and proliferation.[31] Future studies will therefore be needed to determine whether nestin is used to evaluate the functional ability of EPC through differentiation process.

V. CONCLUSION

The results of this study provided the first evidence that nestin was used as a new differentiation marker of EPC. But our studies showed that hypoxic stimuli did not directly affect nestin gene expression in HUVEC.

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< ABSTRACT (IN KOREAN)>

태아 내피전구세포 (endothelial progenitor cell)의 분화과정과 제대정맥 내피세포 (human umbilical vein endothelial cell, HUVEC)의 저산소 배양과정에서 Nestin의 발현양상

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목적: 제 4형 중간형 미세단백질로 알려진 nestin은 내피세포의 혈관증식 및 혈관신생 능력을 평가하는 표지자로 사용할 수 있음이 알려져 있다. 이런 nestin에 대해서 연구를 통해서, 최근 우리는 정상산모에서보다 전자간증 산모의 태반에서 nestin이 더 많이 발현함을 발표하였다. 제대혈액내의 내피전구세포는 혈관증식, 기관형성, 손상된 혈관의 치료에 중요한 역할을 한다고 알려져 있다. 본 연구의 목적은 내피전구세포의 분화과정과 제대정맥 내피세포의 저산소 배양과정에서 nestin 발현의 변화를 확인하고자 하는 것이다.

연구대상 및 방법: 임신 제 37주부터 40주 사이에 분만된 15명의 산모를 대상으로 50 ml의 제대혈액 및 제대를 태아 분만 직후 획득하였다. 내피전구세포는 제대혈액 내에서 단핵세포를 얻은 후, 이 중 섬유결합소 (fibronectin)에 부착된 세포만을 획득하여 7일간 배양한다. 7일간의 배양 후, 이 세포들은 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (acLDL-DiI)과 FITC-labeled Ulex europaeus agglutinin-1 (UEA-1)을 이용한 형광발현을 통하여 내피전구세포임을 확인하고, 다시 15일간의 분화 과정을 거쳐서 증식내피세포 (outgrowth endothelial cell, OEC)로 분화가 유도된다. 제대정맥 내피세포 (human umbilical vein endothelial cell, HUVEC)는 M199 배양액을 통해 배양을 하며, 정상 산소 배양을 위해서는 5% CO_2와 95%의 공기가 사용 되었고, 저산소 배양을 위해서는 5% CO_2와 94%의 N_2 그리고 1%의 O_2가 사용 되었다. HUVEC의 배양은 24시간 유지되었다. 내피전구세포, 증식내피세포, 제대정맥 내피세포에서의 nestin 유전자 발현을 확인하기 위해 semi-quantitative RT-PCR (sqRT-PCR)이 사용되었다.

결과: Nestin 유전자는 내피전구세포에서 발현되지 않았으나, 증식내피세포로 분화되면서 유전자 발현이 나타났으며, 제대정맥 내피세포

에서도 증식내피세포와 비슷한 양상으로 nestin 유전자의 발현이 확인되었다. 제대정맥 내피세포를 24시간 저산소 배양한 결과, nestin 유전자의 발현 양상은 변하지 않았다.

결론: 본 연구에서 nestin은 내피전구세포의 새로운 분화 표지자로 사용될 수 있는 가능성을 보여주었다. 그리고, 제대정맥 내피세포에 있어서 저산소 자극이 직접적으로 nestin 유전자의 발현을 조절하지는 않는 것을 확인할 수 있었다.

핵심 되는 말 : Nestin, 내피전구세포, 제대정맥 내피세포