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Cobalt chloride enhances angiogenic potential of CD133$^+$ cells

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1. ABSTRACT

Umbilical cord blood-derived CD133$^+$ cells differentiate into endothelial cells and induce new blood vessel growth. Hypoxia-inducible factor-1 (HIF-1), a regulator of hypoxia or hypoxia-mimetic agent, activates the SDF-1/CXCR4 signaling pathway and plays an important role in angiogenesis *in vivo*. In this study, we determined whether CD133$^+$ cells enhance angiogenic potential through hypoxia or hypoxia mimetic agent ($CoCl_2$) *in vitro*. The CD133$^+$ cells were cultured respectively, in normoxia (20% O_2), or hypoxia (10% O_2, 3% O_2), or with various concentrations of $CoCl_2$ (50 microM/L, 100 microM /L, 200 microM /L). We showed here that both hypoxia and $CoCl_2$ induced hypoxia cause a significant increase of CD133$^+$ cell migration, proliferation, and tubule-like structure formation. Under conditions of hypoxia or $CoCl_2$ induced hypoxia, the HIF-1α, SDF-1, and VEGF protein and gene expression level are elevated as compared to those under normoxia conditions. These data suggest that a hypoxia mimetic agent can be used to enhance the angiogenic potential of CD133$^+$ cells.

2. INTRODUCTION

Ischemic disorders such as cardiovascular disease, peripheral vascular disease, and stroke have been the leading cause of mortality all over the world. However, the therapies currently available could only delay the progression of disease. Ischemia-driven neovascularization is a crucial element in the recovery of organ function due to the development of collateral vessels, which in turn lead to an increase of blood flow, reducing the size and severity of tissue necrosis (1, 2). Recent studies have indicated that portions of ischemic neovasculature derive from vasculogenesis, whereby endothelial progenitor cells (EPCs) settle at sites of injury, leading to the formation of new vessels (3, 4). The discovery of EPCs has introduced the potential of cellular therapy as a novel approach to the treatment of ischemic disorders. However, only a very small number of transplanted EPCs are recruited in the ischemic tissue (5), thus the efficiency of cell therapy is greatly constrained (6). Consequently, the discovery of a technique capable of enhancing the recruitment and retention of transplanted EPCs is crucial to the regenerative potential of EPCs.

It is widely accepted that hypoxia is the farthest environmental signal in ischemic tissue. It has also been demonstrated that increases in hypoxia-inducible factor (HIF)-1 activity mediate the adaptive responses to hypoxia/ischemia. The stromal cell-derived factor-1a (SDF-1a) and its corresponding receptor CXCR4 are up-regulated and play a prominent role in the recruitment of EPCs (7). Several groups have exploited the SDF-1–CXCR4 axis and have examined the migration of blood-derived stem cells to the ischemic tissue for increasing neovascularization for recovery of organ function. It has been shown that forced expression of SDF-1 in the heart by either adenoviral gene delivery (8) or by controlled release of SDF-1a through PEGylated fibrin patches could improve the c-kit$^+$ cell settlement and attenuated ischemic cardiomyopathy (9).

Previous studies have shown that during normoxia, the HIF-1 proteins are continuously degraded by a ubiquitin- and proteasome-dependent pathway, whereas HIF-1α proteins accumulate rapidly during hypoxia. Hypoxia-mimetic agents, such as cobalt chloride ($CoCl_2$), act by blocking degradation and induce the accumulation of hypoxia-inducible factor-1alpha (HIF-1α) protein (10). Thus, we hypothesized that $CoCl_2$ could enhance the EPCs therapy by mimicking a hypoxic microenvironment.

EPCs, which are isolated from bone marrow, umbilical cord blood, or peripheral blood using CD34, CD133 or KDR antigens, possess high potential of proliferation, form *de novo* vessels upon implantation *in vivo*, and spontaneously anastomose with host vasculature (11). The CD133$^+$ epitope is considered a hematopoietic stem cell marker which is expressed on EPCs, but not on mature endothelial cells (12-15). Some previous studies demonstrate that a subpopulation of these cells can differentiate into mature endothelia cells (16-18) and form new blood vessels *in vivo* (19). However, the function of the CD133$^+$ cells was not clear, especially under the condition of hypoxia.

In this study, the CD133$^+$ cells selected from umbilical cord blood were cultured respectively in enviromental hypoxia and $CoCl_2$ induced hypoxia, and subsequently their proliferation, differentiation, and angiogenic ability were compared. Based on this experiment, we explored whether the mechanism of the augmentation of EPC neovascularization was mediated by $CoCl_2$ via the SDF-1/CXCR4 signal axis.

3. MATERIALS AND METHODS

3.1. Human CD133$^+$ cell isolation

We obtained human umbilical cord blood samples from healthy donors. Diluted human umbilical cord blood samples were layered on a Ficoll-Hypaque1.077 (Pharmacia) gradient and centrifuged at 500*g* for 20 min at room temperature. The mononuclear cells (MNCs) sampled from the buffy coat layer were washed with phosphate bicarbonate saline solution containing 1% bovine serum albumin (PBS/BSA). The CD133$^+$ cell fraction was then isolated from the MNCs. A total of 1×10^8 MNCs were mixed with 100µl fragment crystallizable receptor (FcR), blocking reagent, and 100µl of anti-human CD133$^+$ antibody (MACS, Miltenyi Biotec, Germany) and incubated for 30 min at 4°C. The CD133$^+$ cells were then isolated utilizing a MACS separator (VarioMACS, Miltenyi Biotec, Germany) with LS$^+$/VS$^+$ column (MACS, Miltenyi Biotec, Germany).

3.2. Cell culture

Next, the isolated CD133$^+$ cells were cultured in EGM-2MV Microvascular Endothelial Cell Growth Medium-2 with Bulletkit supplement (5% FBS, hydrocortisone, gentamycin, VEGF, human FGF, human EGF, R3-IGF, and ascorbic acid) (Clonetics, LONZA, USA) in a culture dish. The medium was changed every three to four days. The isolated cells were exposed to either the conditions of hypoxia or to the conditions of normoxia. The oxygen microenvironment was controlled using airtight chambers (Billups–Rothenberg) with oxygen concentrations of 20%, 10%, and 3%. Additionally, HIF-1α stabilizer $CoCl_2$, at the concentration of 50µmol/l $CoCl_2$, 100µmol/l $CoCl_2$, and 200µmol/l $CoCl_2$, induced the conditions of hypoxia. All experiments were conducted in a 37°C incubator.

3.3. Acetylated LDL uptake and labeling of UEA-1 lectin binding sites

Initially, CD133$^+$ cells were cultured in 6-well plates and incubated for 4h at 37°C. The cells were then cultured in endothelial growth media containing 10 µg/ml Dil-Ac-LDL (Molecular Probes) in a 5% CO_2 environment for 30 days, at which time the cells were washed with PBS and fixed in 2% paraformaldehyde for 10 min. Cells were then incubated with 10µg/ml of UEA-1 (SIGMA) for 1h at 4°C. Cell imaging was visualized by fluorescence microscopy (Leica, Germany) in which five randomly selected microscopic fields per well were used to calculate the number of double-positive cells.

3.4. Tubule formation

We aimed to assess the activity level of the formation of tube-like structures. The isolated CD133$^+$ cells were seeded on 24-well cell culture plates pre-coated with Matrigel (BD Bioscience) at 2×10^4 cells/well and maintained in EGM-2MV. The cells were cultured for 24h at 37°C and 5% CO_2. The tube-like structures were visualized using a light microscope after 24h, and five fields per group were assessed by an independent examiner in a blinded manner.

3.5. Flow cytometric analysis of cell surface markers

Using flow cytometry we identified the isolated CD133$^+$ cells and evaluated the cell differentiation based on markers CD34, CD133, CD31, and KDR (20, 21). Initially, the attached, isolated CD133$^+$ cells were subjected to flow cytometric analysis. Subsequently, the CD133$^+$ cells were cultured under various oxygen tensions and various $CoCl_2$ concentrations as described above for seven days. Next, 1×10^6 cells were trypsinized, washed twice with a PBS solution containing 2% BSA, and incubated with monoclonal antibody CD34-FITC (Miltenyi Biotec, Germa), CD133-PE (Miltenyi Biotec, German), CD31 (Chemicon), and KDR-APC (R&D, USA) at 4°C for 30 min. The cells were then stained with the CD31 antigen and reincubated with the FITC-labeled secondary antibody for

Table 1. Primers used for real time polymerase chain reaction amplification

Primer	Forward primer	Reverse primer
HIF-1α	5′-AGTGTACCCTAACTAGCC-3′	5′-CACAAATCAGCACCAAGC-3′
VEGF	5′- CTTGCCTTGCTGCTCTAC-3′	5′- GATGTCCACCAGGGTCTC-3′
SDF-1	5′-CTGGGCAAAGCCTAGTGAAG-3′	5′-CTCCCAGAAGAGGCAGACC-3′
β-actin	5′-AAATCGTGCGTGACATTAA-3′	5′-CTCGTCATACTCCTGCTTG-3′

20 min at 4°C. After two washes, the cells were analyzed by flow cytometry (FACS, Beckton Dickinson, USA).

3.6. Migration assay

The $CD133^+$ cell migration was evaluated with chemotatic factors, at the various oxygen tensions and various $CoCl_2$ concentrations described above, using a Millicell-PCF chamber. The cells (5×10^5), in a volume of 300μl, were placed into the upper chamber of a 8μm pore size, 24-well millicells plate (Millipore). Nearly all of the cells remained on the lower side of the membrane after migration, thus quantification was performed by simply counting these cells. The plates were then placed at 37°C, 5% CO_2 for 24h. After migration, the non-migrating cells were removed from the upper chamber of the millicells using cotton-tipped applicators, and the membranes of millicells filters were fixed with 2% PFA. Cells that had migrated to the bottom of the millicells were stained with Diff-Quik and we counted the number of migrated cells by randomly choosing five microscopic fields (200x) in each chamber and averaging the number of migrated cells.

3.7. Cell wound healing

$CD133^+$ cells were plated in 6-well dishes and cultured at 37°C until they reached confluency. Cell monolayers were scraped using a sterile 200μl yellow plastic tip to produce small wounds of similar size. Wounded monolayers were then washed with phosphate-buffered saline to remove cell debris, and complete EGM-2MV was added. Cells were incubated at 37°C under various oxygen tensions and various $CoCl_2$ concentrations as described above for differing lengths of time to evaluate cell migration (0 and 20h following scratch formation). Digital images were obtained at each experimental time point. Images were analyzed, and wound lengths were quantified using Image Pro Plus software (Version 6.0).

3.8. Cell proliferation assay

The proliferation of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were cultured in 96-well plates (BD) at a density of 6×10^3 cells/well, with each well containing 150μl of culture medium with either various concentrations of $CoCl_2$ or fresh culture medium under various oxygen tension conditions. Following incubation for 24–96h MTT (Sigma, USA), dissolved in phosphate buffered saline (PBS) and filtered, was added to each well at a final concentration of 5mg/ml. The cells were incubated at 37°C for 4h. The water-insoluble dark blue formazan crystals were dissolved in DMSO. The optical density was measured at a wavelength of 570 nm using a SPECTRAMAX 190 microplate reader (Molecular Devices, USA). All experiments were performed in triplicate. The average absorbance value for each group was calculated and represented the cell viability.

3.9. Measurement of VEGF, FGF, and HIF-1α protein by ELISA

The supernatant samples were collected and assessed for bFGF and VEGF using an ELISA kit according to the manufacturer's instructions (R&D systems). As a control, basal media was also analyzed. The absorbance was measured at 450 nm. Total HIF-1α was extracted from all cells. Additionally, total HIF-1α protein was extracted from all cells after ELISA detection, in which a total HIF-1α kit (R&D Systems) was utilized according to the manufacturer's instructions. The concentration of HIF-1α was calibrated using a HIF-1α standard curve and activated HIF-1α was measured from the nuclear extract. After the lysate samples were centrifuged at 16,000×g at 4°C for 10 min, the supernatant samples were collected. To measure the activity of HIF-1α, 50 μg nuclear extract/well samples were incubated with biotinylated double-stranded (ds) oligonucleotide containing a consensus Duo-set ELISA mouse active HIF-1α kit binding site (R&D Systems). The activity of HIF-1α was measured by 450 nm optical density.

3.10. Real time PCR

Total RNA was extracted from 1×10^7 $CD133^+$ cells using trizol reagent (Invitrogen). Samples of RNA were reverse transcribed to cDNA using a Superscribe First-Strand synthesis system (Invitrogen). The cDNA samples were used as the template for PCR in order to verify the expression levels of HIF-1α, SDF-1, and VEGF at the transcription stage. Thermocycling conditions of VEGF and SDF-1 were as follows: 45 sec at 94˚C for 35 cycles, 45 sec at 51.5˚C for 35 cycles, and 45 sec at 72˚C for 35 cycles, with a final 10-minute-extension at 72˚C. Thermocycling conditions of HIF-1α and β-actin (which served as the control) were as follows: 30 sec at 94°C for 35 cycles, 45 sec at 51.5°C for 35 cycles, and 45 sec at 72°C for 35 cycles, with a final extension for 10 min at 72°C. The primers are listed in Table 1.

3.11. Western blot analysis

A total of 2×10^6 $CD133^+$ cells from each of the different groups were washed with PBS, followed by total protein extraction and collection. The proteins collected from each group were separated on a 7.5% SDS-Tris glycine PAGE gel. The blots were blocked with 5% non-fat milk in TBS with 0.05% Tween-20 (TBST) and probed with rabbit anti-human polyclonal antibody HIF-1α (1:200; SANT CRUZ), VEGF (1:200; SANT CRUZ), SDF-1 (1:200; SANT CRUZ), or GAPDH antibody overnight at 4°C. Membranes were then washed and probed with horseradish peroxidase-conjugated secondary antibody at a concentration of 1:1000 (SANT CRUZ). The labeled bands from the washed blots were visualized using enhanced chemiluminescence (ECL). Protein expression was semi-quantified using a Tiannen imager and analysis system.

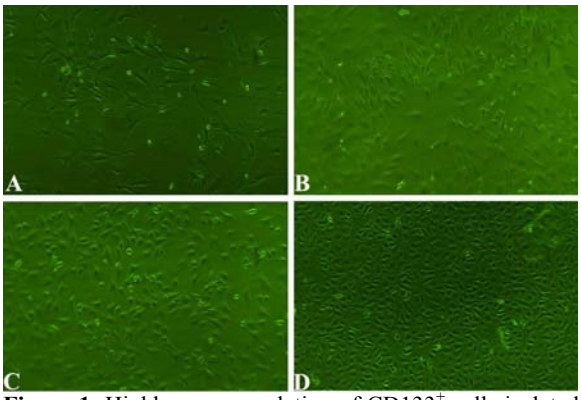

Figure 1. Highly pure population of $CD133^+$ cells isolated from umbilical cord blood cells. A, B, C, D represents the cells on the 2nd, 4th, 6th and 8th day after isolation.

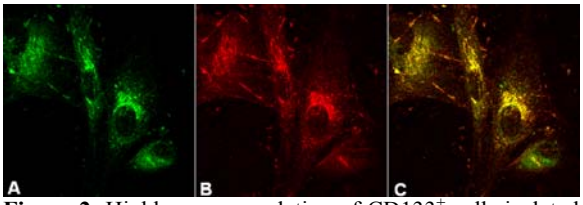

Figure 2. Highly pure population of $CD133^+$ cells isolated from umbilical cord blood cells. Representative $CD133^+$ cells that bind to Ulex europaeus agglutinin-1 (A, green fluorescence) and endocytose acetylated low-density lipoprotein (B, red fluorescence), C, (yellow/orange) dual labeled cells.

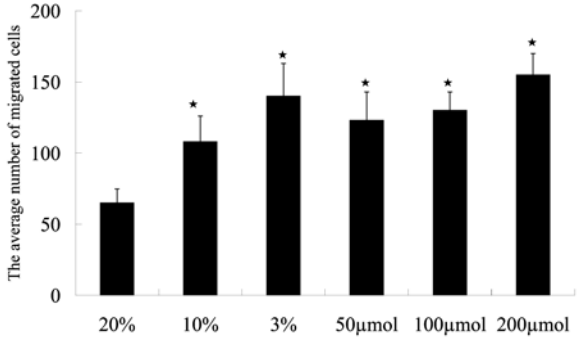

Figure 3. The average number of migrated cells at 20%, 10%, and 3% oxygen level and 50µmol/l $CoCl_2$, 100µmol/l $CoCl_2$, and 200µmol/l $CoCl_2$ concentration.

3.12. Statistical analysis

Data are expressed as mean ± SEM. The differences between the normoxia and hypoxic groups were analyzed by one-way ANOVA. Differences with a $p<0.05$ were considered to be statistically significant.

4. RESULTS

4.1. Characteristics of isolated CD133 positive cells from umbilical cord blood

Following three days of isolation, CD133 positive cells exhibited rapid proliferation and were adherent to the culture dishes and retained an adherent, spindle-like shape. Subsequently, these cells continued to proliferate and the typical cobblestone formation appeared following the seventh day after adherence was observed, suggesting endothelial differentiation (Figure 1). In order to evaluate the biological characteristics of the $CD133^+$ cells, the cells were examined for the uptake of acetylated LDL and UEA-1, thus, we visualized the cultured cells with double positive FITC-UEA-1 staining to measure the uptake of DiI-ac-LDL (Figure 2). To identify the isolated $CD133^+$ cells among the human umbilical cord blood cells, the positive expression of cell surface markers CD34, CD133, KDR, which is a generally accepted method to determine EPCs (22) and CD31 positive cells, was observed using FACS analysis. The fraction of attached cells cultured for 3 days exhibited high expression of CD34, CD133, KDR, and CD31 and exhibited 96.7%, 96%, 95.2%, and 0.1% positive results, respectively. Under the various oxygen tensions and various $CoCl_2$ concentrations described above, we found a more substantial decrease in the positive percentage of CD34, CD133, and KDR AT cells in the hypoxia groups and various $CoCl_2$ concentration groups compared to the normoxia group; moreover, the percentage of CD31 positive AT cells was significantly higher in the treated groups compared to the normoxia group, evidence of cell differentiation.

4.2. Cell migration

Human umbilical cord blood isolated $CD133^+$ cell migration properties were investigated at three distinct oxygen tensions (3%, 10%, and 20% O_2) and various $CoCl_2$ concentrations (50µmol/l, 100µmol/l, 200µmol/l), and a statistically significant increase under hypoxia conditions compared to conditions of normoxia was revealed. Cell migration was visibly confirmed by imaging the bottom of the millipores stained with Diff-Quik. The number of migrating $CD133^+$ cells under various oxygen gradients was 59.6±3.89 (20%), 100.8±3.38 (10%), and 141.1±4.64 (3%), whereas the number of migrating $CD133^+$ cells under various $CoCl_2$ concentrations was 120.7±4.7 (50µmol/l), 131.2±4.23 (100µmol/l), and 154.5±3.52 (200µmol/l) (Figure 3). When exposed to conditions of hypoxia or hypoxia conditioned media, $CD133^+$ cells exhibited a significant increase in migration compared to $CD133^+$ cells under normoxia conditions, indicating that the cells exhibited enhanced chemotaxis under hypoxia conditions. Moreover, the lower the oxygen concentration or the higher the $CoCl_2$ concentration, the greater the number of migrating cells. Additionally, as a chemotatic factor, $CoCl_2$ demonstrated a greater degree of chemotaxis. Additionally, the cell wound assay allowed for an estimation of the level of $CD133^+$ cell migration. Under various conditions, hypoxia as a chemokine significantly accelerated the recovery of the exposed surface area (Figure 4). In the chemotaxis assay, an identical phenomenon was detected in the wound healing assay. Taken together, these results suggest that the conditions of hypoxia or $CoCl_2$ act as a chemokine and potentially enhance endothelial cell migration.

4.3. Proliferation

The MTT assay was used to examine the proliferative capacity of $CD133^+$ cells under different conditions. Compared to the conditions of normoxia,

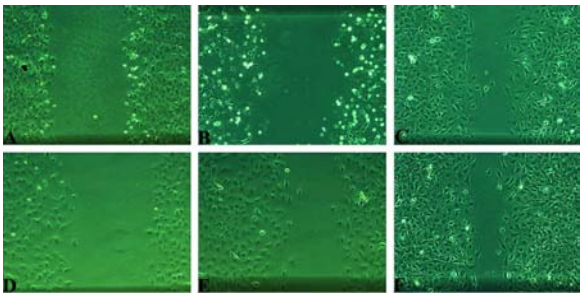

Figure 4. A-F 20 hours after scratch formation at a 20%, 10%, and 3% oxygen level and in a concentration of 50μmol/l $CoCl_2$, 100μmol/l $CoCl_2$, and 200μmol/l $CoCl_2$, respectively.

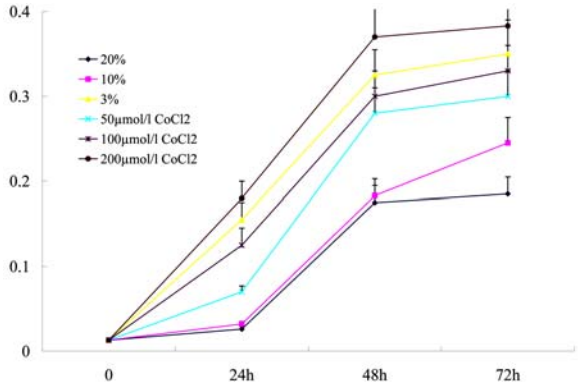

Figure 5. Proliferation of $CD133^+$ cells at varying oxygen tensions and different concentrations of $CoCl_2$.

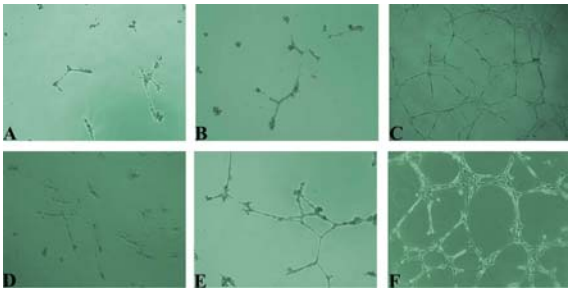

Figure 6. Effects of hypoxia on tubule formation. (A-C) Images of tubules formed under the oxygen concentrations of 3%, 10%, and 20%, respectively. (D-F) Images of tubules formed under the concentrations of 50μmol/l $CoCl_2$, 100μmol/l $CoCl_2$, and 200μmol/l $CoCl_2$, respectively.

$CD133^+$ cell proliferation significantly increased as oxygen tension decreased (Figure 5). Between 24 and 96h of incubation, the $CoCl_2$ concentration enhanced the proliferation of $CD133^+$ cells in a dose-dependent manner, whereas oxygen tension inhibited cell proliferation in a dose-dependent manner.

4.4. Tubule formation ability

The ability to form tubules in the matrigel is a hallmark of EPC behavior. For the tubule formation assay, $CD133^+$ cells were plated onto a layer of matrigel and incubated for 24 h. The ensuing tubule activity of the cells was observed on the matrigel. The conditions of hypoxia and hypoxia conditioned media significantly enhanced the tubule formation of $CD133^+$ cells, whereas under normoxia conditions the tubule-like structures were difficult to observe. Morphological differences were clearly observed for tubules generated by $CD133^+$ cells at various oxygen tensions and various $CoCl_2$ concentrations (Figure 6). Specifically, the cells cultured in 200μmol/l $CoCl_2$ produced well-rounded, circular tubules with borders typically comprising more than one cell. Tubules formed at 3% O_2 and 50μmol/l and 100μmol/l $CoCl_2$ exhibited a more polygonal shape, and were typically bordered by a one-cell layer. The tubule-like structures were less likely to be fully enclosed in the cells cultured in 10% O_2 and under normoxia conditions.

4.5. Secretion of growth factors by $CD133^+$

The release of different growth factors was used to measure the secretion activity of $CD133^+$ cells. Hypoxia and exposure to $CoCl_2$ induced an increase in the accumulation of selected growth factors such as VEGF, FGF, and HIF-1α compared to normoxia conditions (Fig.7). The cells cultured in 3% O_2 and 200μmol/l $CoCl_2$ exhibited a significant increase of secretion activity compared to the cells cultured under normoxia conditions. The hypoxia-conditioned media induced an increase in the secretion of growth factors in a dose-dependent manner; whereas various oxygen tensions exhibited the opposite effect.

4.6 Hypoxia induced protein expression

The HIF-1α protein expression level, a sensitive marker of hypoxia, was significantly increased in cells that were exposed to conditions of hypoxia compared to cells cultured under normoxia conditions. Quantitative analysis of HIF-1α by Western blot demonstrated an increase of 1.06, 2.68, and 5.22 fold over the control in the HIF-1α protein cultured at concentrations of 50μmol/l, 100μmol/l, and 200μmol/l $CoCl_2$, respectively, and an increase of 2.03 and 4.32 fold over the control in the HIF protein cultured in conditions of 3% and 10% oxygen, respectively (Figure 8). SDF-1 expression was mediated by HIF-1α. $CD133^+$ cells cultured at 3% O_2 expressed an elevated level of SDF that was similar to the level found for cells cultured in the condition of 200μmol/L $CoCl_2$. However, cells cultured at 20% O_2 revealed a lower SDF-1 expression level that was similar to cells cultured in 50μmol/l $CoCl_2$. VEGF was downstream of SDF-1, and we found that the expression of VEGF was associated with the level of SDF-1 expression (Figure 8).

4.7 HIF-1α and downstream gene expression

It is well understood that the HIF-1α pathway is activated when cells are exposed to a lower level of oxygen tension. The gene expression of $CD133^+$ cells under hypoxia conditions and $CoCl_2$ hypoxia-conditioned media was compared to the gene expression level in normoxia using RT-PCR. Hypoxia led to elevated HIF-1α mRNA levels. HIF-1α levels increased in a dose-dependent manner with $CoCl_2$ concentrations and correlated with the

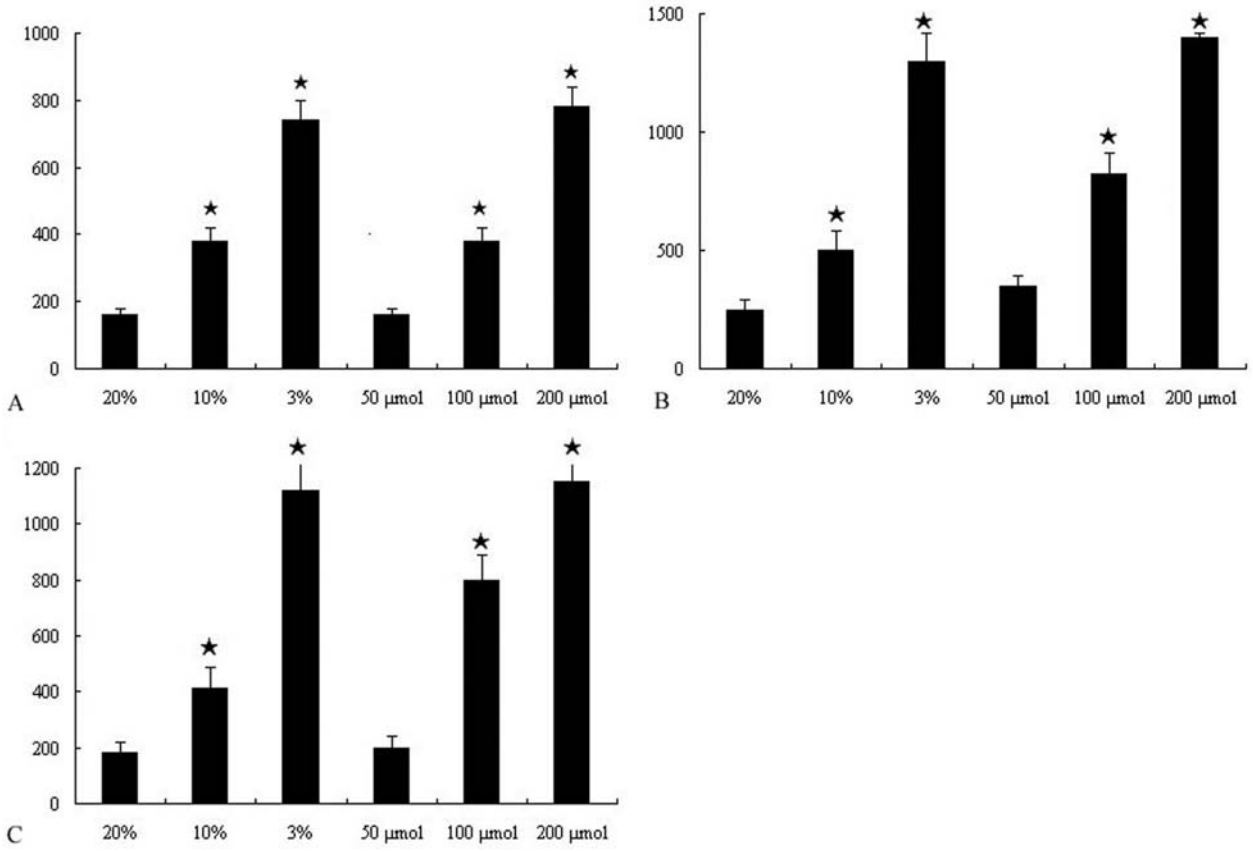

Figure 7. ELISA analysis of expressed VEGF, FGF, HIF-1α at a 20%, 10%, and 3% oxygen level and at concentrations of 50μmol/l $CoCl_2$, 100μmol/l $CoCl_2$, and 200μmol/l $CoCl_2$, respectively.

decrease of oxygen tension, with 1.06, 12.3, and 10.8 fold increases found in the conditions of 50μmol/l, 100μmol/l, and 200μmol/l $CoCl_2$, respectively, and increases of 0.2 in 10% O_2 and 0.31 in 3% O_2 compared with 0.12 in conditions of normoxia. SDF-1 is regulated upstream by HIF-1α, thus mRNA levels of SDF-1 were assessed. A corresponding SDF-1 increase in expression was observed under hypoxia and $CoCl_2$ conditions compared with normoxia conditions (Figure 9). This is in concordance with previous findings that SDF-1 expression may be mediated by a HIF-1α-dependent pathway. In addition, chemokine up-regulation was accompanied by paralleled VEGF upregulation. The expression of VEGF mRNA correlated with the level of HIF-1α, and increased under exposure to hypoxia and $CoCl_2$ hypoxia conditioned media compared with normoxia.

5. DISCUSSION

Hypoxia-inducible factor (HIF-1) is an oxygen-dependent transcriptional activator that facilitates the adaptation of cells and tissues to low O_2 concentrations (22, 23). HIF-1 is a heterodimeric protein complex composed of two subunits: a stable and constitutively expressed HIF-1β and a hypoxically inducible subunit HIF-1α (24, 25). HIF-1α plays a key role in controlling erythropoiesis, glycolysis, and angiogenesis (24, 26). Under normoxia, HIF-1α proteins are rapidly degraded, resulting in essentially no detectable HIF-1α protein (27). In the absence of oxygen, hydroxylation is inhibited because of substrate (O_2) deprivation and HIF-1α accumulates.

Activation of HIF activity, a master regulator of angiogenesis, produces a more physiological and 'balanced' angiogenic response than the use of a single growth factor for therapeutic angiogenesis and HIF activity plays a positive role in responding to organ or tissue damage (28). For example, the elevated level of HIF-1α and VEGF could induce the formation of new blood vessels, thereby providing increased blood flow and oxygen supply and reducing the harmful response to ischemia (24). Effective vascular remodeling following ischemic injury is dependant upon an integrated program of HIF-dependent gene expression. HIF-1 serves as an overall mediator of the angiogenic response to hypoxia by inducing multiple angiogenic factors, including VEGF and angiopoietins (29). The importance of HIF-1 in neovascularization suggests that it potentially has useful clinical implications, and the regulation of HIF-1 provides numerous opportunities for therapeutic intervention.

Cobalt, a prolyl hydroxylation inhibitor, mimics hypoxia and stabilizes HIF-1 (23, 30), which enables blocking of degradation and induces the accumulation of

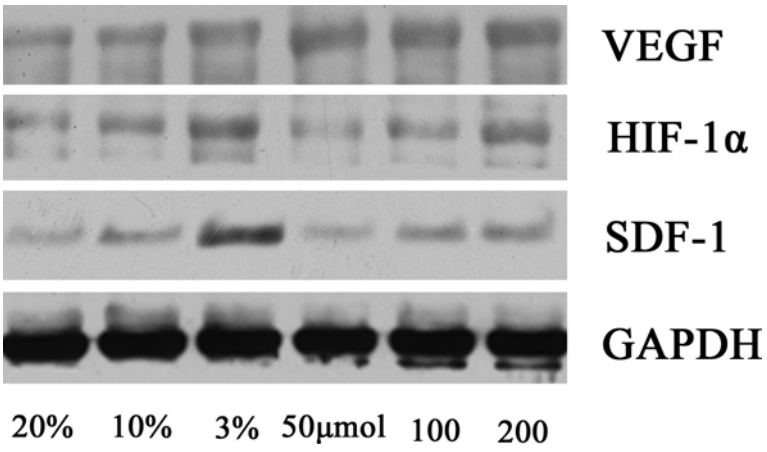

Figure 8. Western blot analysis of expressed VEGF, HIF-1α, SDF-1 at 20%, 10%, and 3% oxygen level and at concentrations of 50μmol/l $CoCl_2$, 100μmol/l $CoCl_2$, and 200μmol/l $CoCl_2$, respectively.

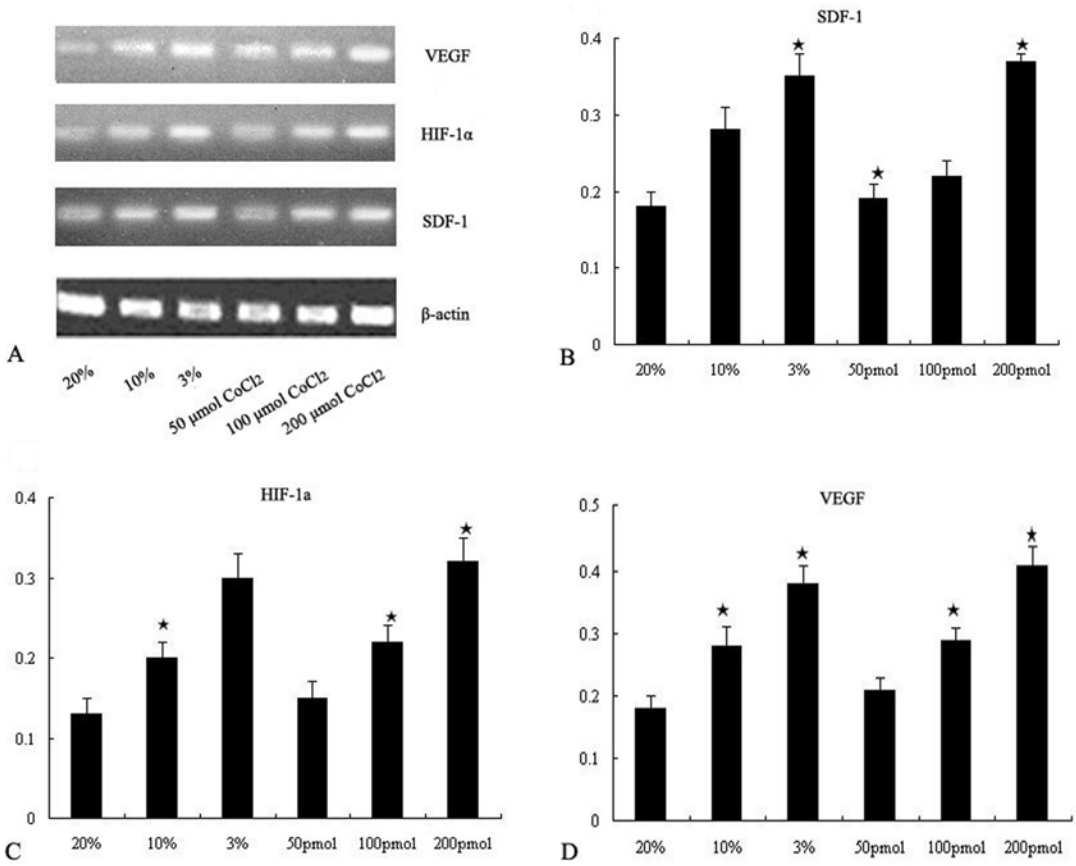

Figure 9. Analysis of real-time RT-PCR revealed mRNA expression of VEGF, SDF-1, and HIF-1α.

hypoxia-inducible factor-1alpha (HIF-1α) protein (9). In our study, the results demonstrated that $CoCl_2$ induced HIF-1 expression both at the gene level and protein level in dose-dependent manner. The concentration of HIF-1α in the culture medium and the $CD133^+$ cell HIF-1α mRNA levels were significantly elevated in the 200μmol/l $CoCl_2$ induced hypoxia condition compared to the normoxia group. Additionally, the expression of HIF-1α decreased as oxygen tension increased under three distinct oxygen tensions. These results are in concordance with previous reports that suggest HIF-1α expression increases in response to hypoxia as well as in response to the HIF-1 mimic Cobalt, which activates the signaling pathway of SDF-1-CXCR4 under normoxia conditions (7).

During the course of this study, we reported several methods utilized to improve the expression of HIF-1α: vectors transduced via electro-poration (31), transgenic animals (32), or adenoviral transduction (33). It has been previously demonstrated that macrophage-derived peptide PR39 stabilizes HIF-1α by decreasing its degradation (34). Direct induction of HIF-1 has been achieved using the N- or C-terminal of ODDD polypeptides that block VHL-mediated degradation (35). Targeting of proline and asparaginyl hydroxylases may additionally serve as potential strategies for increasing HIF activity (36). Gene therapy alone may not provide a sufficient dose to allow for a significant response in young and middle aged mice (37). Pharmacologically, $CoCl_2$ provides a superior, safer method that could potentially increase HIF-1α mRNA and protein expression in cutaneous wounds of diabetic Leprdb/db mice (38). There is an evident increase in HIF-1α protein levels, HIF-1 DNA-binding activity, and HIF-1 transcriptional activity upon exposure of cells to $CoCl_2$.

Over-expression of HIF-1α leads to EPC differentiation into ECs, affects the proliferation index and expression of EC markers like CD31 (39, 40), and causes endothelial cells to exhibit sprouting and tubule formation *in vitro* thereby preventing EPC apoptosis (41, 42). Our study demonstrated that $CD133^+$ cells exposed to $CoCl_2$ *in vitro* exhibited increased proliferation in a dose-dependent manner; a large number of $CD133^+$ cells exposed to $CoCl_2$ and conditions of hypoxia expressed EC-lineage markers CD31 compared to normoxia-conditioned $CD133^+$ cells. This result suggested that hypoxia or preconditioning hypoxia enhanced the differentiation of $CD133^+$ human umbilical cord blood derived cells into endothelial cells. The release of VEGF and SDF-1 from $CD133^+$ cells was significantly increased in a dose dependent manner. SDF-1 and VEGF-A exerted a synergistic effect (43, 44) and played an important role in the differentiation process. In the tubule formation assay, the $CD133^+$ cells under conditions of 200μmol/l $CoCl_2$ induced hypoxic conditions produced well-rounded, circular tubules with borders typically comprised of more than one cell to a greater extent than the cells under 3% oxygen conditions and the other groups. This suggested that cobalt up-regulates SDF-1 by stabilizing HIF-1, thus enhancing the angiogenic properties of EPCs through angiogenic factors (8, 45-47). These results correlate with a previous finding that hypoxia may enhance the angiogenic properties of EPCs through up-regulation of several genes involved in angiogenesis, such as SDF-1, VEGF, the VEGF receptors KDR and FLT-1, and bFGF (7, 8, 45-47), all of which, as the experimental results indicate, play an important role in angiogenesis *in vivo* and contribute to the formation of capillary-like structures *in vitro*.

Stromal cell-derived factor-1 (SDF-1) has been identified as a hypoxia-responsive element that contains binding sites for hypoxia-inducible factor-1 (HIF-1) in bone marrow stromal cells (48), and is a central transcriptional regulator of hypoxia-specific gene expression; its receptor, CXCR4, is expressed on progenitor cells (41). The SDF-1–CXCR4 axis plays a pivotal role in the retention and homing of hematopoietic stem cells in the bone marrow stem cell niche (49). The mobilization and homing of bone marrow mononuclear cells and progenitor cells by VEGF or hypoxia are both mediated via the SDF-1-CXCR4 axis. We found that the SDF-1 and VEGF mRNA and protein levels also increased in a dose-dependent manner with $CoCl_2$ concentration; $CoCl_2$ exhibited a strong ability for chemotaxis, and the number of $CD133^+$ cells that migrated to the media with $CoCl_2$ increased upon exposure to higher concentrations of $CoCl_2$. Additionally, in the cell wound healing assay, the conditions of hypoxia or $CoCl_2$ as a chemokine led to a significantly accelerated recovery of the exposed cell surface area. This suggested that HIF-1 signaling is required for the upregulation of SDF-1 in response to hypoxia or HIF-1 mimics, Cobalt, and activates the signaling pathway of SDF-1-CXCR4 under normoxia conditions. Cobalt up-regulates SDF-1 by stabilizing HIF-1 (7), thus it is a potential agent for progenitor cell mobilization and recruitment to ischemic tissues by increasing serum SDF-1 (50, 51). SDF-1 and VEGF potentially mediate the mobilization and migration of bone marrow-derived stem and progenitor cells (50-56). G-CSF, a clinically important agent (57), also induces mobilization in a time and dose-dependent manner. G-CSF, which mobilizes marrow cells, increases serum SDF-1 concentrations while effecting SDF-1 degradation in marrow, hence SDF-1/CXCR4 axis signaling may represent a common pathway to mobilization of bone marrow cells.

HIF-1 is a critical mediator that regulates the expression of genes encoding angiogenic cytokines, such as VEGF, placental growth factor (PLGF), stromal-derived factor (SDF)-1, Ang-2 and Ang-4, and their cognate receptors VEGFR1, VEGFR2, and CXCR4 (7, 47, 58). In this study, the $CD133^+$ cells under $CoCl_2$ treatment conditions expressed VEGF, SDF-1 and FGF, and exhibited enhanced proliferation, differentiation and tubule formation. VEGF and SDF-1 induced angiogenesis via the SDF-1/CXCR4 axis to mobilize and promote angiogenesis. These results suggested that cobalt chloride as a HIF-1 mimetic induces the condition of hypoxia, upregulates SDF-1 by stabilizing HIF-1, enhances angiogenic cytokine expression, and improves the angiogenesis of $CD133^+$ cells *in vitro*. Recent studies suggest that pro-angiogenic factors such as VEGF and basic FGF upregulate CXCR4 and SDF-1 expression on endothelial cells (42, 59).

6. CONCLUSION

In summary, hypoxia-mimetic stimuli with $CoCl_2$ resulted in an increase in $CD133^+$ cell differentiation *in vitro*; exposure to $CoCl_2$ enhanced cell proliferation, differentiation, and tubule sprouting and formation; cells exhibited a greater ability to migrate via HIF-α independent mechanisms, and the expression of SDF-1 and VEGF was altered.

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Abbreviations: HIF-1: Hypoxia-inducible factor-1; SDF-1a: stromal cell-derived factor-1a; EPCs: endothelial progenitor cells; MNCs: mononuclear cells, PBS: phosphate bicarbonate saline, FcR: fragment crystallizable

receptor; ECL: enhanced chemiluminescence; PLGF: placental growth factor

Key Words: $CD133^+$ cells, Hypoxia; SDF-1, Cobalt Chloride, Umbilical Cord Blood

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