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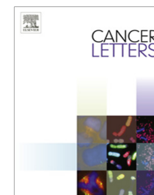

Generation of erythroid cells from fibroblasts and cancer cells *in vitro* and *in vivo* ☆,☆☆

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ABSTRACT

Bone marrow is generally considered the main source of erythroid cells. Here we report that a single hypoxia-mimic chemical, $CoCl_2$, can increase the size of fibroblasts and cancer cells and lead to formation of polyploidy giant cells (PGCs) or polyploidy giant cancer cells (PGCCs), activation of stem cell marker expression, increased growth of normal and cancer spheroid, and lead to differentiation of the fibroblasts and epithelial cells toward erythroid lineage expressing hemoglobins both *in vitro* and *in vivo*. Immunohistochemical examination demonstrated that these cells are predominantly made of embryonic hemoglobins, with various levels of fetal and adult hemoglobins. Ectopic expression of c-Myc induced the generation of nucleated erythoid cells expressing variable levels of embryonic and fetal hemoglobins. Generation of these erythroid cells can be also observed via histological examination of other cancer cell lines and human tumor samples. These data suggest that normal and solid cancer cells can directly generate erythroid cells to obtain oxygen in response to hypoxia and may explain the ineffectiveness of conventional anti-angiogenic therapies for cancer, which are directed at endothelium-dependent vessels, and offer new targets for intervention.

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1. Introduction

Oxygen plays a critical role in energy production, and organisms including tumor cells have developed a programmed response to hypoxia that increases glucose utilization and stimulates erythropoiesis and angiogenesis to compensate for the decrease in available oxygen [1]. Erythropoiesis, the process by which erythroid cells are produced, is stimulated by decreased O_2 in circulation. However, in humans with certain diseases, erythropoiesis also occurs outside the bone marrow, within the spleen or liver [2]. Erythroid cells consist mainly of hemoglobin, a complex metalloprotein containing heme groups whose iron atoms temporarily bind to oxygen molecules in the lungs and release them throughout the body via vessels [3]. Hemoglobin is the iron-containing oxygen-transport metalloprotein in erythroid cells. Hemoglobin molecules consist of four globin chains, each of which has a heme moiety attached. Different hemoglobins have different capacities for oxygen affinity and release. In humans, mature erythroid cells are oval and flexible biconcave disks. They lack nucleus and most organelles, allowing maximum space to accommodate hemoglobin [4,5]. There are high-level expressions of Hb Gower-1 ($\zeta2\varepsilon2$), Hb F ($\alpha2\gamma2$), and Hb A ($\alpha2\beta2$) during the embryonic, fetal, and adult developmental stages, respectively [6,7]. Human α and β globins are expressed at moderate levels in developing embryos and fetuses, whereas various amounts of embryonic ζ and ε globins and their encoding mRNAs can be detected in fetal erythroid cells and in adult-stage reticulocytes, respectively [8]. Intact erythroid cells containing a complex mixture of embryonic, semi-embryonic, and fetal hemoglobins have been shown to bind O_2 strongly [9].

While the bone marrow as a source of hematopoietic cells, it has been shown that human embryonic stem cells and induced pluripotent stem cells with a combination of one or more transcription factors can also generate these cells *in vitro* [10–13]. Szabo et al. demonstrated the ability to generate multilineage blood progenitors from human dermal fibroblasts without establishing pluripotency [11]. Furthermore, erythroid differentiation can be induced

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in K-562 cells by different kinds of chemical reagents, including hemin [14], butyric acid [15,16], 5-azacytidine [17], and chromomycin and mithramycin [18]. Bianchi et al. reported that human leukemic K562 cells can be induced *in vitro* to erythroid differentiation by cisplatin; they found that differentiation of K562 cells is associated with an increase in the expression of embryo–fetal globin genes [19]. These findings open a new possibility that the fibroblasts can also serve as an alternative source for hematopoietic cells, although this idea has not been tested vigorously at different experimental settings.

Hypoxia is a key regulator in stem cells, erythroid differentiation, angiogenesis, and tumor development [20] and is associated with the formation and maintenance of cancer stem cells [21,22]. Cobalt chloride ($CoCl_2$) has been widely used as a hypoxia mimic to treat aplastic anemia and renal anemia [23,24]. Here we report that ovarian fibroblasts and cancer cells can directly generate hemoglobin and erythroid cells *in vitro* and *in vivo* using hypoxia mimic $CoCl_2$. Our study provides a novel insight how normal and neoplastic tissue can obtain O_2 during normal tissue and tumor development.

2. Materials and methods

2.1. Cell culture and generation of immortalized cell lines

Fresh specimens of human fallopian tube fimbria and ovarian tissue were obtained from patients at The University of Texas MD Anderson Cancer Center under a protocol approved by the Institutional Review Board. Culture of primary fallopian tube epithelial cells (FTEs) and normal ovarian fibroblasts (NOFs) was performed as described previously [25]. All FTE and NOF cells were maintained in a 1:1 mixture of medium 199/MCDB 205 (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Intergen), 10 ng/mL epidermal growth factor (Sigma–Aldrich), and 100 U/mL penicillin/streptomycin (Sigma–Aldrich). Primary FTE187, NOF151, and NOF137 cells were infected sequentially with a retrovirus containing pBabe-hygro-hTERT and pBabe-puro-p53 siRNA against mRNA [26]. NOF137p53ihT was infected sequentially with retrovirus containing pLNCX-neo-c-Myc cDNA. FTE187hTERT was infected sequentially with a retrovirus containing pBabe-zeo-SV40 early region and pBabe-puro-HRASV12 as described previously [25]. Infected cells were selected in Zeocin (500 μg/mL), hygromycin B (100 μg/mL), and puromycin HCl (1 μg/mL) for 5–10 d following each of the respective rival infections. MDA-MB-231, and BT-549 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Phoenix, WI-38, and BJ cells were purchased from the American Type Culture Collection and maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.2. Cell treatment with $CoCl_2$

The cells were cultured in medium with FBS and antibiotics until the cells reached 90% confluence. We treated the cells with different concentrations of $CoCl_2$ for different times (Supplementary Table 1). After being rinsed with 1× phosphate-buffered saline (PBS), the cells were cultured in medium with FBS and antibiotics. After cells recovered from $CoCl_2$ treatment, they were cultured with stem cell medium contained 80% DMEM/nutrient mixture F-12, 20% knockout serum replacement (Gibco/Invitrogen), 1% non-essential amino acid, 1 mM L-glutamine (Gibco/Invitrogen), 0.1 mM 2-mercaptoethanol, and 4 ng/ml of basic fibroblast growth factor (Gibco/Invitrogen).

2.3. Immunofluorescent staining of spheroids

The cell lines listed above formed multiple spheroids after treatment with $CoCl_2$ when cultured in stem cell medium. The spheroids were detached gently via pipetting and centrifuged at 400*g* for 5 min to obtain spheroid pellets. The spheroids attached to coverslips after culture with complete medium for several hours. The spheroids were then fixed in ice-cold acetone for 10 min. After washing in Tris-buffered saline and Tween-20 three times for 5 min each, the spheroids were incubated with 1% bovine serum albumin in PBS and Tween-20 for 30 min to block unspecific binding of antibodies. Primary and secondary antibodies in PBS and Tween-20 with 1% bovine serum albumin were added to the coverslips (for detailed antibody information, see Supplementary Table 2) and then incubated in a humidified chamber for 1 h at room temperature. The spheroids were stained with DAPI for 1 min and observed under a fluorescence microscope (Eclipse TE 2000-U; Nikon).

2.4. Surface marker analysis of NOF137p53ihTc-Myc by flow cytometry

To confirm the role of the C-Myc gene in erythroid cell differentiation, the infected NOF137p53ihTC-Myc cells were treated with $CoCl_2$. After $CoCl_2$ treatment, the recovered NOF137p53ihTc-Myc cells produced suspension cells. To characterize the nature of these suspension cells, we spun down the floating NOE137P53ihTc-Myc cells and then resuspended them in PBS buffer with 1% albumin to a concentration of $1–2 \times 10^7$ cells/ml. 50 μl of cell suspension was aliquoted into five tissue culture tubes to directly conjugate with antibodies at concentration suggested by e-Bioscience. The solutions were then mixed gently, incubated for 20 min on ice, washed with 1 × PBS buffer, resuspended in 300–400 μl of buffer, and analyzed by flow cytometry. Five samples including (cells only, CD45 FITC only, CD71 PE only, CD34 APC only, and CD45FITC + CD71 PE + CD34APC) were prepared for flow cytometric analysis.

2.5. Paraffin embedding of blocks of spheroids and floating cells

Media containing the spheroids and floating cells described above were centrifuged at 100*g* for 5 min. The supernatant was removed, 1 ml of 70% ethanol was added to the pellet to fix the spheroids, and 50 μl of eosin was added to the vial containing spheroids and floating cells. The samples of spheroids and floating cells were dehydrated in a graded ethanol series (70%, 80%, 95%, and 100% for 15 min per grade). The vials were then infiltrated with acetone, absolute xylene, a mixture of 50% xylene and 50% paraffin, and purified paraffin at 65 °C for 15 min each. All of these steps were performed in a 1.5-mL vial. The spheroids were then embedded in paraffin and sectioned for hematoxylin and eosin (H&E) staining and immunohistochemical analysis.

2.6. H&E and immunohistochemical staining

For H&E staining, 4-μm sections of formalin-fixed, paraffin-embedded spheroids were deparaffinized and rehydrated and then counterstained with hematoxylin for 1 min and eosin for 2 min. Immunohistochemical staining of the sections was performed by using the avidin–biotin–peroxidase method as described previously [27]. The sections were incubated with primary antibodies overnight at 4 °C in a humidified chamber (Supplementary Table 1 contains detailed antibody information). The nuclei in the sections were counterstained with hematoxylin.

2.7. Spheroid and BT-549 cells injection in nude and NOD.CS17-Prkdc SCID mice

NOF151p53ihT, NOF137p53ihT, and WI-38 spheroids were trypsinized and then centrifuged at 400*g* for 5 min. The supernatant was removed, and 0.1 mL of 1 × PBS was added to resuspend the pellets. The spheroids were mixed with 0.1 mL of PBS buffer and 0.1 mL of Matrigel in syringes and kept on ice before injection. The spheroid–Matrigel mixtures were subcutaneously injected into the flanks of 6- to 8-week-old NOD.CS17-Prkdc SCID mice. Two months later, small nodules (0.2 cm in size) formed in two of four mice injected with NOF151p53ihT spheroids, one of four mice injected with NOF137p53ihT spheroids, and one of four mice injected with WI-38 spheroids. For BT-549 cell injection, BT-549 cells after $CoCl_2$ treatment were subcutaneously injected into the flank of nude mice (1×10^5 per mouse). The mice were killed by cervical dislocation following CO_2 inhalation, and the nodules were removed and fixed in 10% formalin for routine histologic examination and immunohistochemical staining. The care and use of the mice were approved by the MD Anderson Institutional Animal Care and Use Committee.

2.8. Human ovarian tumor samples

The use of human tumors and blocks for immunohistochemical and H&E staining was approved by the MD Anderson Institutional Review Board. For primary culture of human ovarian cancer, fresh human ovarian tumor samples were washed with PBS three times and then sterilized with penicillin and streptomycin. The samples were minced into pieces and cultured with complete Dulbecco's modified Eagle's medium. The medium was changed every 1–2 weeks.

2.9. Smears of supernatants

Supernatants from the primary culture of human ovarian cancer were pipetted out of the flasks and centrifuged at 400*g* for 5 min. Thirty to fifty microliters of 1 × PBS was used to resuspend the pellets. A 10-μl mixture of PBS and cells was dropped onto slides for smearing. The smear slides were fixed with 75% ethanol and then air-dried for H&E staining.

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