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Human mesenchymal stromal cells could deliver erythropoietin and migrate to the basal layer of hair shaft when subcutaneously implanted in a murine model

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ABSTRACT

Mesenchymal stromal cells (MSC) are an attractive cell-targeting vehicle for gene delivery. MIDGE (an acronym for Minimalistic, Immunologically Defined Gene Expression) construct is relatively safer than the viral or plasmid expression system as the detrimental eukaryotic and prokaryotic gene and sequences have been eliminated. The objective of this study was to test the ability of the human MSC (hMSC) to deliver the erythropoietin (EPO) gene in a nude mice model following nucleofection using a MIDGE construct. hMSC nucleofected with MIDGE encoding the EPO gene was injected subcutaneously in Matrigel at the dorsal flank of nude mice. Subcutaneous implantation of nucleofected hMSC resulted in increased hemoglobin level with presence of human EPO in the peripheral blood of the injected nude mice in the first two weeks post-implantation compared with the control groups. The basal layer of the hair shaft in the dermal layer was found to be significantly positive for immunohistochemical staining of a human EPO antibody. However, only a few basal layers of the hair shaft were found to be positively stained for CD105. In conclusion, hMSC harboring MIDGE-EPO could deliver and transiently express the EPO gene in the nude mice model. These cells could be localized to the hair follicle and secreted EPO protein might have possible role in hair regeneration.

1. Introduction

Erythropoietin (EPO) stimulates red blood-cell production and is produced in the fetal liver and adult kidney (Jelkmann, 1992; Fisher, 2003). Since the first successful cloning of the human EPO gene in 1985, recombinant EPO (rhuEPO) has become a therapeutic option for renal anemia in chronic renal failure (CRF) patients and several forms of non-renal anemia. In light of predicted rise in CRF in aging populations globally, the need to develop a cost-effective alternative is apparent (Tsakiris, 2000; Eckardt, 2001). Transplanting EPO-producing cells of human origin is an ideal therapeutic alternative for CRF patients because it would be more cost effective and less likely to result in the serious complications of pure red-cell aplasia due to auto-regulation (Bennett et al., 2004).

The success of producing a biologically functional EPO depends on glycosylation of the EPO protein (Krantz, 1991). The N-glycans, for example, play a major role in secretion, molecular stability, solubility, receptor binding affinity and *in vivo* elimination of EPO (Elliott et al., 2003). The choice of host cells, the culturing conditions and the purification procedures determine the composition of the glycan isoforms of a rhuEPO preparation (Jelkmann, 2008). To date, human EPO gene has also been transfected in myoblast (Hamamori et al., 1995) and skin cells (Gothelf et al., 2010) and has successfully corrected anemia in renal failure in a murine model. Mesenchymal stromal cells (MSC) have also been explored to carry and deliver functional EPO in *in vivo* studies (Bartholomew et al., 2001; Eliopoulos et al., 2003, 2006).

MSC is a type of stem cells that are easy to be isolated, robust in *ex vivo* growth and amenable to genetic modifications (Doering, 2008). Human MSC express intermediate levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I molecules and can be induced by interferon-γ to express HLA class II and Fas ligand; they do not express the co-stimulatory molecules B7-1, B7-2, CD40, or CD40 ligand. They should therefore be recognized by alloreactive T-cells. However, human, baboon, and murine MSC failed to elicit a proliferative response from

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allogeneic lymphocytes. When pre-cultured with interferon-γ for full HLA class II expression, MSC still escape recognition by alloreactive T-cells. Unlike other non-professional antigen-presenting cells, this failure is not reversed by provision of CD28-mediated costimulation. Furthermore, MSC are immunosuppressive and inhibit T-cell alloreactivity induced in mixed lymphocyte cultures or by non-specific mitogens (Tse et al., 2003; Singer and Caplan, 2011).

MSC also possess the capability to migrate to sites of tissue damage or inflammation. This ability to migrate to sites of acute tissue injury has been demonstrated in the setting of bone fracture, cerebral ischemia and in infarcted heart (Caplan and Bruder, 2001; Tomita et al., 2002; Parekkadan and Milwid, 2010). Local delivery of EPO by MSC is clinically useful to rescue damaged tissues from compromised local circulation such as critical hind-limb ischemia, myocardial infarction or spinal cord injury. There are ample animal studies to show that EPO could induce formation of new blood vessels in damaged tissues (Meer et al., 2005; Zhang et al., 2007).

Our previous experiments have shown that human mesenchymal stromal cells (hMSC) isolated from adult bone marrow could be used as a carrier for the EPO gene. The transfected hMSC could stably express the EPO protein as determined by enzyme-linked immunosorbent assay (ELISA) (Mok et al., 2012), leading to induction of differentiation of human hematopoietic stem cells into erythroid colony *in vitro* (Mok et al., 2008). In these studies, transfection was achieved through nucleofection of a new construct, named MIDGE (Minimalistic, Immunologically Defined Gene Expression). MIDGE is a linear, double-stranded DNA consisting solely of the expression cassette, capped with hairpin structures at the ends for protection against exonuclease degradation. The MIDGE construct offers the following advantages: (i) the construct contains only the necessary elements required to express the desired gene with no resistance markers or other unwanted genes, (ii) immune-stimulatory sequences, such as CpGs, are minimized, (iii) the construct eliminates the risk of recombination and mutagenesis of wild-type viruses, and (iv) the construct could be transferred easily as it is smaller in size compared with the plasmid system (Schakowski et al., 2001).

In the current study, we aimed to test the hMSC in carrying and delivering the EPO gene in a MIDGE construct following nucleofection in an *in vivo* system. The nucleofected cells were subcutaneously implanted in the dorsal flank of nude mice and the effects of the expressed EPO protein on the hemoglobin levels in the peripheral blood were studied for two months. The implanted cells expressing EPO protein were found to be localized in the subcutaneous layer of mice.

2. Materials and methods

2.1. Nucleofection of MIDGE-EPO into hMSC

Nucleofection of MIDGE-EPO into hMSC was performed using U-23 pulsing program as previously described (Mok et al., 2008). Generally, 2 μg of MIDGE-EPO was used to nucleofect 5×10^5 hMSC and seeded on 35 mm cell culture petri dishes containing Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin antibiotics (Gibco-Invitrogen, Grand Island, NY). After 24 h, the adherent nucleofected cells were then trypsinized for implantation into nude mice.

2.2. Western blot analysis

To detect the presence of EPO in the supernatant of nucleofected cells, Western blot analysis was performed using a rabbit polygonal anti-human EPO polyclonal antibody (Cat. No. H-162) (Santa Cruz Biotechnology; Santa Cruz, CA). The EPO immunoreactivity was visualized using the WesternBreeze Chromogenic Western Blot Immunodetection Kit (Gibco-Invitrogen). For positive control, rhuEPO (Cat. No. 02625) (Stem Cell Technologies, Vancouver, Canada) was used.

2.3. Implantation of hMSC into mice

In a preliminary study on the sustainability of hMSC in the Matrigel one week post-implantation, a Balb/c mouse was used. Approximately 5×10^5 hMSC were stained using the PKH-26 red fluorescent cell linker mini kit (Sigma–Aldrich, St. Louis, MO, USA) according to the protocol recommended by the manufacturer. These cells were then washed with phosphate buffered saline (PBS) by centrifuging the cells at $200 \times g$ for 10 min, suspended in 50 μl of PBS and mixed with 0.5 ml thawed BD Matrigel Matrix High Concentration (Cat. No. 356231) (BD Biosciences, Bedford, MA) at 4 °C. The Matrigel mixture was then injected subcutaneously into the left flank of the mouse. At body temperature, the Matrigel would rapidly acquire a semisolid form.

To study the delivery of EPO protein by the nucleofected hMSC, experiments were performed on 12-week old Nu/Nu male mice (BioLASCO, Taipei, Taiwan) with protocols approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (PP/PAT/2008/AINOON/12-AUGUST/227-JAN-2009-DEC-2011). Six mice were used for each group of experiment: mice implanted subcutaneously with Matrigel mixed with PBS, mice implanted with non-nucleofected hMSC encapsulated in Matrigel and mice implanted with nucleofected hMSC (hMSC-EPO) encapsulated in Matrigel. Approximately 5×10^5 cells were injected into the subcutaneous tissues of the nude mice according to the protocol stated above. Non-nucleofected cells were prepared by nucleofecting cells without MIDGE-EPO.

2.4. Determination of human EPO and hemoglobin levels in the blood:

Under general anesthesia, 100 μl of blood was withdrawn from the retro-orbital venous plexus of the mice using heparinized capillary tube (Hirschmann Laborgerate, Eberstadt, Germany) and transferred into a 0.5 ml ethylenediaminetetraacetic acid (EDTA) tube (Greiner Bio-One GmBH, Kremsműnter, Austria). The tube containing the blood sample was then centrifuged at $400 \times g$ for 20 min to obtain the plasma for human EPO measurement using the Human Erythropoietin ELISA Immunoassay Kit (Stem Cell Technologies). Hemoglobin measurement was performed according to the procedures described in Choudhri et al. (1997).

2.5. Immunohistochemical staining on Matrigel implants and other organs

At 2 and 8 weeks post-implantation, the mice were sacrificed by cervical dislocation to harvest the implant, together with the skin and the muscle layer. The harvested implants were fixated and sections of 3 μm were prepared for hematoxylin and eosin (H&E) (Sigma–Aldrich), and immunohistochemical staining using the LSAB+ System-Horseradish Peroxidase Kit (Dako Cytomation, Glostrup, Denmark). For the detection of human EPO and CD105, anti-human rabbit EPO (Cat. No. sc-7956; dilution 1:200) (Santa Cruz Biotechnology) and anti-human mouse CD105 (Cat. No. 555690; dilution 1:200) (BD Biosciences Pharmingen, San Diego, CA) primary antibodies were used. To localize hMSC in other major organs, the lung, heart, spleen, kidney, liver and brain were also harvested on the second and eight weeks, and stained according to the above procedure.