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Wound-healing markers after autologous and allogeneic epithelial-like stem cell treatment

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Abstract

Background aims. Several cytokines and growth factors play an essential role in skin regeneration and epithelial-like stem cells (EpSCs) have beneficial effects on wound healing in horses. However, there are no reports available on the expression of these growth factors and cytokines after EpSC therapy. *Methods.* Wounds of 6 cm^2 were induced in the gluteus region of 6 horses and treated with (i) autologous EpSCs, (ii) allogeneic EpSCs, (iii) vehicle treatment or (iv) untreated control. Real time polymerase chain reaction was performed on tissue biopsies taken 1 and 5 weeks after these treatments to evaluate mRNA expression of *interferon (IFN)-γ*, *interleukin (IL)-6*, *vascular endothelial growth factor* (*VEGF*), *epidermal growth factor* (*EGF*), *insulin-like growth factor (IGF)-1* and *epidermal keratin* (*eKER*). *Results.* One week after treatments, mRNA levels of *IL-6* ($P = 0.012$) and *VEGF* ($P = 0.008$) were higher in allogeneic EpSC-treated wounds compared with controls. Also, mRNA levels of *IGF-1* were higher at 1 week in both autologous ($P = 0.027$) and allogeneic ($P = 0.035$) EpSC-treated wounds. At week 5, all EpSC- and vehicle-treated wounds demonstrated significantly higher *IFN-γ*, *VEGF* and *eKER* mRNA expression compared with controls and compared with their respective levels at week 1. *Conclusions.* Equine wounds treated with allogeneic EpSCs demonstrate a significant increase in mRNA expression of *IL-6*, *VEGF* and *IGF-1* in the acute phase. In the longer term, an increase in *IFN-γ*, *VEGF* and *eKER* mRNA was detected in the wounds treated with allogenic EpSCs, autologous EpSCs or their vehicle.

Key Words: *allogeneic, autologous, cytokines, growth factors, horse, skin, stem cells*

Introduction

The skin is the largest organ of the mammalian body, and its healing and regeneration has been extensively studied [1]. Currently, scientists mainly focus on enhancing skin wound repair because wounds might result in severe dysfunction and can be life threatening when they are chronic or involve an extended skin surface [2]. In general, wound healing consists of a dynamic process driven by cell proliferation and differentiation and is mediated by different types of growth factors, cytokines and chemokines [3,4].

It has been reported in horses that autologous as well as allogeneic epithelial-like stem cells (EpSCs) improve different wound-healing parameters, resulting in significantly enhanced wound repair [5,6]. The authors described a significant increase in early cellular immune response and vascularization to result in reduced tissue granulation and earlier wound closure. This is reinforced by other studies, which have reported that an earlier inflammatory peak in wounds of ponies leads to enhanced wound contraction and epithelialization compared with wounds in horses [7–9]. Additionally, it has been described that keratinocytes would also be able to induce a cellular immune response after exposure to pro-inflammatory cytokines, such as interferon (IFN)-γ [10,11] which results in enhanced wound healing as well [12].

Besides inflammatory parameters, growth factors also play a pivotal role during the wound-healing process. Vascular endothelial growth factor (VEGF), for example, promotes skin wound angiogenesis by exerting a paracrine effect on endothelial cells [13], and epidermal growth factor (EGF) influences epithelial

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cell proliferation and migration and enhances angiogenesis [14,15]. Another important growth factor is insulin-like growth factor (IGF), which promotes keratinocyte migration [16], and research has shown that wounds with lower IGF levels display less healing capacity [17]. Additionally, epidermal keratin (eKER) is an interesting structural parameter to investigate because this fibrous protein can be found in wool, hair, nails, mammalian claws, equine and bovine hooves and horns [18]. Indeed, these intermediate filaments are abundantly present in stratified epithelia, particularly in the suprabasal layers of the epidermis, and may therefore be considered an epidermal reconstitution marker [19]. The present study is the first to investigate the aforementioned growth factors, cytokines and keratin mRNA expression levels at two time points in an *in vivo* experimental wound-healing study where the following treatments are evaluated: (i) autologous EpSCs, (ii) allogeneic EpSCs, (iii) vehicle treatment and (iv) untreated control wounds.

Methods

Skin sampling for EpSC isolation and wound induction

Six French trotter mares between 5 and 7 years of age were included in this study. A 1-cm^2 skin sample was retrieved from the neck region of these horses for EpSC isolation and characterization as previously described [20]. Twelve weeks after EpSC harvesting, 12×6 cm^2 wounds were created in the gluteus region of all horses to allow the evaluation of diverse treatments (three wounds per treatment group) at different time points, as reported by our group [5]. Before skin harvesting, horses were sedated with detomidine (0.04 mg/kg intravenous [IV]; Medesedan), and analgesia was achieved using butorphanol (0.1 mg/kg IV; Dolorex). Procaine 4% plus adrenalin was used for local subcutaneous anesthesia. Samples for real-time polymerase chain reaction (rt-PCR) analyses were taken at 1 and 5 weeks after treatment by means of a 3-mm punch biopsy after aforementioned sedation and analgesic drug administration and with a subcutaneous anesthesia consisting of procaine 4% without adrenaline. The experimental procedure was approved by the ethics committee of Global Stem Cell Technology (EC_2012_002, EC_2013_003 and EC_2014_001) and the Faculty of Veterinary Medicine, Ghent University (EC_2014_020).

Different treatment groups

Four treatment groups were considered: (i) autologous EpSCs, (ii) allogeneic EpSCs from two randomly chosen donors within the same group of horses, (iii) Dulbecco's Modified Eagle's Medium (DMEM) as a vehicle control and (iv) untreated controls. Twenty minutes after wound induction, 4×10^6 cells in 2 mL DMEM were injected subcutaneously in the wound margins and 4×10^6 cells in 1 mL DMEM were applied topically for the autologous and allogeneic treatment. Two of the six horses received half the doses in both the autologous and allogeneic treated group because the obtained number of cells for autologous treatment in these horses was insufficient. In the vehicle control wounds, 2 mL DMEM was injected subcutaneously, and 1 mL DMEM was applied topically. The remaining group of control wounds was left untreated to monitor the normal healing process. The horses did not receive any other medication.

RNA isolation and gene expression analysis

Total RNA extraction was performed using Trizol (Life Technologies) reagent following the manufacturer's instructions. RNA was quantified on a Nanodrop (Thermo Scientific) spectrophotometer and a complementary single strand DNA (cDNA) was synthesized from 2 µg of purified RNA to perform rt-PCR using the ABI 7500 Real Time PCR system (Applied Biosystems). The relative expression of the following genes was used to evaluate pro-inflammatory cytokines *IFN-γ* and *IL-6*; growth factors *VEGF*, *EGF* and *IGF-1*; and epidermis reconstitution marker *eKER*.

Each sample was tested in triplicate, and untreated skin was used as a calibrator sample. Real-time conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Wells contained 30 µL of PCR mixture (SYBR Green PCR Master Mix, Applied Biosystems), including 3 µL of cDNA at a dilution of 1:10. The 2-ΔΔct method was used to analyze and normalize the RNA expression of the target genes with respect to the endogenous housekeeping genes *GAPDH* (glyceraldehyde-3- phosphate dehydrogenase) and *18S*. The value Δct was calculated as the difference between the average Ct for each target gene and the *GAPDH*/*18S* genes. The value ΔΔct was obtained as the difference between the average ΔCT for each treatment and the calibrator sample. PCR primers were designed using Primer Express 3.0 software (Applied Biosystems). All primer sequences used in the present study are listed in Table I. Wherever possible, primers were designed to span introns in the genomic DNA to minimize non-specific fluorescence signals due to contaminating genomic DNA.

Statistical analysis

Normally distributed data were expressed as the mean ± SEM. Normality of the data was confirmed using the Kolmogorov-Smirnov test (a = 5%). Statistical analyses were performed using the paired