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3D-Wound healing model: Influence of morphine and solid lipid nanoparticles

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ABSTRACT

For efficient pain reduction in severe skin wounds, topically applied opioids may be a new option. Moreover, by stimulating keratinocyte migration opioids may also accelerate wound healing. Yet, conventional formulations failed to consistently provide sufficient pain control in patients which may be due to local drug degradation or insufficient concentrations at the target site. After having excluded major morphine glucuronidation by keratinocytes and fibroblasts, we next aimed for an optimised formulation. Since long intervals for painful wound dressing changes are intended, the formulations should allow for prolonged opioid release and should not impair the healing process. We developed morphine-loaded solid lipid nanoparticles (SLN, mean size about 180 nm), and tested improvement of wound closure in a new human-based 3D-wound healing model. Standardised wounds were induced by CO_2-laser irradiation of reconstructed human full-thickness skin equivalents (EpiDermFT^{TM}). Morphine, morphine-loaded and unloaded SLN accelerated reepithelialization. Keratinocytes almost completely covered the dermis equivalent after 4 days, which was not the case when applying the vehicle. In conclusion, acceleration of wound closure, low cytotoxicity and irritation as well as possible prolonged morphine release make SLN an interesting approach for innovative wound management.

1. Introduction

Wound management in burn and skin graft patients is still challenging clinicians. Skin wounds are very painful. In particular, wound dressing changes are problematic because of severe pain and the risk to remove the regenerating epithelia. Thus, wound dressing changes are preferably performed in long intervals—ideally after a minimum of 7 days (Cuignet et al., 2005, 2004). Opioids are the gold standard for pain treatment in burn or skin graft patients. Yet, systemic opioid use can be accompanied by side effects such as respiratory depression, nausea and constipation. Opioid receptors on sensory nerve endings, keratinocytes, fibroblasts and melanocytes (Bigliardi-Qi et al., 2004; Bigliardi et al., 1998; Hassan et al., 1993; Salemi et al., 2005; Stein et al., 2009) may provide targets for pain reduction and improved healing by topical morphine application to wounded skin. Until today, however, only small clinical studies and case series have reported a decline in the intensity of pain (Flock, 2003; Platzer et al., 2005; Twillman et al., 1999; Zeppetella et al., 2003) when applying opioid gel formulations. Large-scale controlled clinical studies are still missing which may indicate problems with reproducible efficacy. This could result from local opioid inactivation, inadequate drug delivery or a negative influence of the acidic environment linked to the inflammation.

Importantly, while aiming at local pain reduction, the opioids should not interfere with wound closure to allow for a completely beneficial therapy. Results of preclinical trials on wound healing are contradictory. An important step in wound closure is the reepithelialization of the wound by keratinocytes migrating from the margins to the center of the wound. Both the endogenous opioid β-endorphin and exogenous morphine induce keratinocyte migration in cell cultures (Bigliardi et al., 2002, 2003; Wolf et al., 2009). In addition, met-enkephalin as well as morphine accelerate wound healing in vivo in the rat (Poonawala et al., 2005;

Abbreviations: BSA, bovine (serum) albumin; DMEM, Dulbecco's modified Eagle's medium; 2(3)D, two (three) dimensional; HepG2, human hepatoma cell line; HPLC, high performance liquid chromatography; LD, laser diffractometry; MEME, minimum essential medium Eagle; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PCS, photon correlation spectroscopy; PI, polydispersity index; r.t., room temperature; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; SLN, solid lipid nanoparticle(s); YHWAZ, tyrosin 3-monooxygenase/tryptophan 5-monooxigenase activation protein, zeta-polypeptide.

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Vinogradov et al., 1987). Enhanced cell migration is due to opioid-induced stimulation of nitric oxide production and is not inhibited in acidic environments (Wolf et al., 2009). However, depending on the chronicity of the wound and the stage of the wound healing process, retarded wound healing has been observed in a rodent model (Sassani et al., 2003). A standardised, human-derived disease model is lacking.

Moreover, there is a clear lack of information on the biotransformation of opioids in human skin and the relevance of local activation and deactivation of drugs and other xenobiotics has become obvious only recently (Baron et al., 2001; Chouinard et al., 2008; Lombardi Borgia et al., 2008; Luu-The et al., 2007; Oesch et al., 2007). With respect to morphine, hepatic glucuronidation leading to the formation of morphine-6-glucuronide and morphine-3-glucuronide is the most important pathway of opioid metabolism (for review see: Tegeder et al., 1999). UDP-glucuronosyl transferase 2B7 (UGT2B7) is the relevant isozyme (Coffman et al., 2003; Thorn et al., 2009) and its activity as well as UGT2B7 promotor variants influence the morphine metabolite/morphine ratio following oral morphine administration (Darbari et al., 2008; Tateishi et al., 2003). Plasma concentrations of morphine, morphine-6-glucuronide and morphine-3-glucuronide vary widely, both in healthy subjects and in patients suffering from severe pain (e.g. in burn injuries) (Perreault et al., 2001). Besides the liver, UGT2B7 analogs have also been detected in skin, mammary glands, uterus and ovary (Chouinard et al., 2008), yet the level of expression in human epidermis and reconstructed human epidermis (Episkin®) appears to be low (Luu-The et al., 2007).

To enable topical opioid application to wounds the respective formulation has to be very well tolerated and has to function well under clinically established treatment regimens, e.g. first changes of wound dressings after 1 week. For both aspects, solid lipid nanoparticles (SLN) appear to be carriers of choice. SLN can be built from lipids generally recommended as safe (GRAS status) and well tolerated. Tensids are only needed in low concentrations for particle stabilization (Müller et al., 2007). The irritant potential for skin and eye as well as cytotoxicity for normal human keratinocytes is low (Wolf et al., 2009). Most importantly, SLN can release the loaded agent for days (Jenning et al., 2000a; Wissing et al., 2004; zur Mühlen et al., 1998).

Here we describe a standardised three-dimensional (3D) wound model obtainable by defined laser-induced destruction of reconstructed full-thickness skin. In addition, we examine the influence of morphine while also considering possible metabolism by local glucuronidation.

2. Materials and methods

2.1. Materials

EpiDermFT™ full thickness skin models (EFT-400, diameter 12 mm) were delivered from MatTek Corporation (Ashland, MA, USA) along with the maintenance and assay medium. Human hepatoma cells (HepG2) were purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Medium for the HepG2 cells (minimum essential medium Eagle, MEME), phosphate buffered saline (PBS, pH 7.4), bovine serum albumin (BSA) and materials for the haematoxylin–eosin staining were purchased from Sigma–Aldrich (München, Germany). Culture medium for keratinocytes and supplements were obtained from Lonza (Walkersville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and L-glutamine were purchased from PAA Laboratories (Cölbe, Germany), fetal calf serum and non-essential amino acids from Biochrom (Berlin, Germany). Tissue freezing medium was from Leica (Nussloch, Germany).

For RNA extraction NucleoSpin® RNA II from Macherey-Nagel (Düren, Germany) was used. Amplification grade DNAse I, amplification buffer and stop solution for DNA digestion were obtained from Sigma–Aldrich. The reverse-transcription kit was obtained from Fermentas (St. Leon-Rot, Germany). The SYBR Green I Masterplus kit for RT-PCR was purchased from Roche Diagnostics (Mannheim, Germany).

Morphine hydrochloride was purchased from Fagron (Barsbüttel, Germany). Compritol® 888 ATO (glyceryl behenate) was a gift from Gattefossé (Weil a. Rh., Germany). Lutrol F68® (poloxamer 188) was obtained from BASF (Ludwigshafen, Germany).

2.2. Cell culture

Primary human keratinocytes and fibroblasts were isolated from juvenile foreskin, the remainder of circumcision surgeries or from surplus female breast skin obtained from plastic surgery (with permission). Keratinocytes were grown in the keratinocyte basal medium supplemented with epidermal growth factor, insulin, gentamicin sulphate, amphotericin B, hydrocortisone and bovine pituitary extract (keratinocyte growth medium). Fibroblasts were grown in the fibroblast growth medium consisting of DMEM, 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown to a confluence of 70%, respectively (Gysler et al., 1997). Keratinocytes of the second or third passage and fibroblasts of the second to fourth passage were used for the experiments.

HepG2 cells were maintained in 75 cm^2 flasks (Nunc, Wiesbaden, Germany) with MEME containing 2 mM L-glutamine, 1% non-essential amino acids, 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were expanded at 37 °C and 5% CO_2 and the medium was changed every 2 or 3 days. HepG2 cells up to the 25th passage were used for the experiments.

2.3. Gene-expression of UGT2B7

UGT2B7 mRNA expression in primary human keratinocytes and fibroblasts was tested using human hepatoma cells (HepG2) as positive control (Lancon et al., 2007). Experimental protocols followed the manufacturer's instructions.

RNA extraction: 4×10^5 cells were incubated for 24 h at 37 °C, 5% CO_2 in a six-well-plate. Total RNA isolated using NucleoSpin® RNA II was dissolved in 60 μl of nuclease-free water and was stored at −80 °C. Total RNA amount and purity were tested by UV spectroscopy (wavelength setting: 260 and 280 nm). Additionally, integrity and purity of the RNA were evaluated by gel electrophoresis.

cDNA-synthesis: Potentially contaminating genomic DNA was digested by the incubation of 0.5 μg RNA (in 8 μl water) with 1 μl amplification buffer (10-fold concentrated) and 1 μl amplification grade DNAse I at room temperature for 15 min. 1 μl of stop solution was added and the mixture was incubated at 70 °C for 10 min. The DNA-free RNA was stored on ice.

For the reverse transcription 9 μl of a master mix containing 4 μl reaction buffer (5-fold concentrated), 1 μl ribolock RNAse inhibitor, 2 μl 10 mM dNTP mix, 1 μl RevertAid M-MuLV reverse transcriptase and 1 μl random hexamer primer were added to the RNA. The mixture was incubated at 25 °C for 5 min, followed by 60 min at 42 °C. Finally the mixture was heated up to 70 °C for 5 min. The obtained cDNA was diluted with RNAse-free water 1:4 and stored at −80 °C until use.

Real-time polymerase chain reaction (RT-PCR): For a relative quantification of UGT2B7 mRNA expression, 2 μl of cDNA-dilution were analyzed in a LightCycler® 480 Real-Time PCR System