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Research paper

Influences of opioids and nanoparticles on in vitro wound healing models

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ABSTRACT

For efficient pain reduction in severe skin wounds, topical opioids may be a new option – given that wound healing is not impaired and the vehicle allows for slow opioid release, since long intervals of painful wound dressing changes are intended. We investigated the influence of opioids on the wound healing process via in vitro models, migration assay and scratch test. In fact, morphine, hydromorphone, fentanyl and buprenorphine increased the number of migrated HaCaT cells (spontaneously transformed keratinocytes) twofold. In the scratch test, morphine accelerated the closure of a monolayer wound (scratch). As possible slow release application forms are nanoparticulate systems like solid lipid nanoparticles (SLN) and dendritic core-multishell (CMS) nanotransporters, we evaluated the effect of unloaded nanoparticles on HaCaT cell migration, too. CMS nanotransporters did not inhibit migration, SLN even enhanced it (twofold). Applying morphine plus unloaded nanoparticles reduced morphine effects possibly due to uptake into CMS nanotransporters and adsorption to the surface of SLN. In contrast to SLN, TGF-β1 was taken up by CMS nanotransporters, too. Both nanoparticles are tolerable by skin and eye as derived from Episkin-SM™ skin irritation test and HET-CAM assay. No acute toxic effects were observed either. In conclusion, opioids as well as the investigated nanoparticulate carriers conform the essential conditions for topical pain reduction.

1. Introduction

Agreeable wound management for burn and skin graft (donor site) patients is challenging clinicians. To avoid systemic side effects and the – though limited – risk of dependency, long lasting pain reduction by topical application of opioids appears advantageous. Due to the peripheral expression of (μ) opioid receptors on nociceptive nerve endings [1,2], this should be possible by principle, yet clinical success appears limited [3,4], which may be due to a low amount of opioid reaching the target site over time. Since nanoparticulate carrier systems can increase skin penetration severalfolds [5–8] and can slowly release the loaded drug [9–12], we aimed to investigate the potential of nanoparticulate carriers for a topical application of opioids. First, however, we had to unravel the influence of either drug or carrier on wound healing in order to exclude negative effects. In fact limited data indicate that opioids may even enhance wound healing [13,14].

The wound healing process consists of at least three different phases. The initial inflammatory phase is mainly characterized by a clotting process and chemotaxis of inflammatory cells which help to cleanse the wound. Reepithelialization occurs in the proliferative phase. Stimulated by cytokines, keratinocytes migrate across the wound matrix to close the wound before scar formation is taking place [15]. Enhancement of keratinocyte migration indicates a positive effect on the healing process, delayed migration indicates a negative effect. In the final remodelling phase we observe a reorganization of the scar tissue. Here we studied the effects of opioids on human keratinocyte migration using migration assay and scratch test.

For the use in severely damaged skin the choice of pharmaceutical formulation is very important, as the wound healing process must not be disturbed by the vehicle constituents either. Since the change of the wound dressing is very painful, long lasting analgesic effects conforming a low changing-frequency would be optimal. Aiming for nanocarrier systems for opioid delivery we

Abbreviations: B, buprenorphine; BSA, bovine serum albumin; CMS, dendritic core-multishell nanotransporters; $c_{particle}$, particle concentration; F, fentanyl; FCS, fetal calf serum; H, hydromorphone; HaCaT, human adult low calcium temperature keratinocytes; M, morphine; n.s., not significant; PBS, phosphate buffered saline; r.t., room temperature; SD, standard deviation; SLN, solid lipid nanoparticle(s); TGF-β1, transforming growth factor β1.

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compared solid lipid nanoparticles (SLN) [7,8,16,17] and dendritic core-multishell (CMS) nanotransporters which can encapsulate hydrophilic as well as lipophilic substances and transport them to polar and apolar environments [18,19]. Improvement in skin penetration by CMS nanotransporters even can surmount the efficiency of SLN [6]. SLN and CMS nanotransporters were tested for the influence on keratinocyte migration and local tolerability.

While SLN are composed of lipids having GRAS (generally recommended as safe) status, the local tolerability of CMS nanotransporters is by and large unknown. In fact, due to the nanosize, the carriers may show novel properties compared with the bulk material. Since they are enabled to interact with cells [6], subcellular structures, etc., possibly harmful effects have to be excluded [20], which is of crucial importance especially with the intention to apply the nanotransporters onto skin without any or just recovering barrier function.

2. Materials and methods

2.1. Materials

Morphine hydrochloride, buprenorphine hydrochloride, hydromorphone hydrochloride and naloxone hydrochloride dihydrate were purchased from Fagron (Barsbüttel, Germany). Fentanyl citrate and transforming growth factor-β1 (TGF-β1) were obtained from Sigma–Aldrich (Munich, Germany). Test substances were dissolved in phosphate-buffered saline (PBS, pH 7.4 or pH 6.5, respectively) with 0.4% bovine serum albumin (BSA, Sigma-Aldrich).

Compritol® 888 ATO (glyceryl behenate) was a gift from Gattefossé (Weil a. Rh., Germany). Lutrol F68® (poloxamer 188) was obtained from BASF (Ludwigshafen, Germany). Chemicals for the synthesis of the CMS nanotransporters were purchased from Fluka (Seelze, Germany), except for polyglycerol amine (PG_{10000}) which was prepared as described [21]. Reagents for the diazotization reaction were purchased from Promega (Mannheim, Germany). Fibronectin, Giemsa stain solution and all RPMI 1640 medium and supplements for HaCaT cell culture were purchased from Sigma–Aldrich (Munich, Germany). Culture medium for keratinocytes and supplements was obtained from Lonza (Walkersville, MD, USA). Polycarbonate membrane inserts for the migration assays (pore size: 8 μm) were obtained from Biochrom (Berlin, Germany). Isopropanol, sodium dodecylsulfate, hydrochloric acid, acetic acid, sodium hydroxide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (Munich, Germany). Texapon® ASV 50 was from Cognis (Düsseldorf, Germany).

HaCaT cells were a gift from Prof. Fusenig from the DKFZ, Heidelberg. For skin irritation test Episkin-SM™ reconstructed human epidermis (0.38 cm^2) was delivered from SkinEthic Laboratories (Nice, France), as well as the maintenance and assay medium. The white leghorn chicken eggs for the HET-CAM test were purchased from Lohmann livestock breeding (Cuxhafen, Germany).

2.2. Particle preparation

SLN and CMS nanotransporters (Table 1) were unloaded. SLN (10% lipid), composed of 10% glycerol behenate and 2.5% surfactant (poloxamer 188) were prepared as described [17]. In short, the lipid was melted, an aqueous solution of poloxamer 188 of the same temperature was added and a preemulsion was formed using an ultra turrax. The premix was homogenized by a Lab 60 high pressure homogenizer (APV Gaulin, Lübeck, Germany) at 500 bar for 2.5 min. SLN were stored at 8 °C and used for migration experiments within 1 month, though stable for almost 3 months [17].

The CMS nanotransporters have the empirical formula $PG_{10000}(\text{-}NH_2)_{0.7}(C_{18}mPEG_6)_{1.0}$ and were synthesized using previously described procedures [19]. For testing, CMS nanotransporter dispersions were used at concentrations of 0.5% and 5%.

Table 1
Average size and particle size distribution of the applied nanoparticles, measured via photon correlation spectroscopy and laser diffraction [6].

	Size (nm)	Polydispersity index (PI)
SLN	150-170	⩽0.250
CMS nanotransporters	20–30	⩽0.250

2.3. Parelectric spectroscopy

Aiming to elucidate possible adsorption or incorporation processes, interactions of the nanoparticles with morphine were studied by parelectric spectroscopy (PS). This method and its underlying theoretical model have been described in detail elsewhere [17,22,23].

2.4. Cell culture

HaCaT cells were maintained in 75 cm^2 flasks (Nunc, Wiesbaden, Germany) with RPMI 1640 medium (pH 7.4) containing 10% fetal calf serum (FCS) and L-glutamine (5 mM). Cells were grown at 37 °C and 5% CO_2 and medium was changed every 2 or 3 days. As soon as grown confluent, cells were split (1:10 or 1:15).

Primary human keratinocytes were isolated from juvenile foreskin, the remainder of circumcision surgeries. Keratinocytes were grown in keratinocyte basal medium supplemented with epidermal growth factor, insulin, gentamicin sulfate, amphotericin B, hydrocortisone and bovine pituitary extract (keratinocyte growth medium, KGM) to a confluence of about 70% [24]. Keratinocytes of the second or third passage were used for the experiments.

2.5. Migration assays

Migration assays were performed with HaCaT cells in modified Boyden chambers as described [25–27]. To measure chemotaxis inserts with polycarbonate membranes (pore size: 8 μm) were coated with fibronectin (3 μg/ml in aqua bidest.) for 1 h at 37 °C and placed into 24-well plates (Nunc, Wiesbaden, Germany) which already contained the test substances (opioids, naloxone) or transforming growth factor β1 (TGF-β1, positive control) in the indicated concentrations in supplemented RPMI 1640 medium (pH 7.4). The pure solvent served as negative control. HaCaT cells (2×10^5 cells/well) were seeded into the upper chamber and cells were allowed to migrate for 5 h at 37 °C towards the lower chamber. Then medium was aspired and remaining cells on the upper surface of the membrane were removed with a Q-tip. Cells that had migrated into the membrane were fixed in ice cold ethanol (96%) for 2 min at −18 °C and stained with Giemsa solution (1:10 in water) for 30 min at 37 °C. To test the inhibitory effect of naloxone, cells were preincubated with growth medium containing naloxone (1 μM) for 15 min in their culture flasks before they were used for the migration assay.

Migration in acidic environment was studied using medium (pH 6.5) acidified with concentrated hydrochloric acid. To test the influence of carriers on the migration of HaCaT cells, suspensions of SLN (40 μl) and CMS nanotransporters (40 μl), respectively were added to the cell suspension, that is, to the upper chamber during the assay.

To measure chemokinesis the chemokine gradient was disrupted by adding the opioids or TGF-β1 to the upper as well as to the lower chamber.