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Research paper

Wound healing potential of a dimeric InlB variant analyzed by *in vitro* experiments on re-epithelialization of human skin models

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ABSTRACT

A constitutively dimeric truncated variant of internalin B ($InlB_{321}$-CD), acting as stimulator of the receptor tyrosine kinase MET, was tested for dermal wound-healing potential. Due to a lack of the endogenous MET agonist HGF/SF in chronic wounds, HGF/SF substitution by an $InlB_{321}$-CD-loaded hydrogel might be beneficial in chronic wound therapy.

In this study, $InlB_{321}$-CD in solution and incorporated in a hydrogel was tested for mitogenic effects on immortalized human dermal keratinocytes (HaCaT) with an MTT assay. Cell migration was investigated with a scratch assay on primary keratinocytes (PHK) and on HaCaT. For the latter, scratching needed to be mitomycin C-controlled. $InlB_{321}$-CD effects on a model of human skin were analyzed histologically with respect to viability.

$InlB_{321}$-CD led to dose-dependent proliferative effects on HaCaT cells whereas the equimolar dose of monomeric $InlB_{321}$ did not. Upon hydrogel incorporation of $InlB_{321}$-CD its mitogenic activity for HaCaT cells was maintained thus confirming the hydrogel as a promising drug delivery system. Motogenic effects were shown on both HaCaT and PHK cells. $InlB_{321}$-CD neither possesses cytotoxic effects on the viability of a human skin model nor alters its organotypic cell morphology.

1. Introduction

Dermal wound healing is a complex physiological process which is based on the interplay of various cell types, enzymes, released cytokines and growth factors. Upon dysbalance of any component, wounds may become chronic. The pleiotropic hepatocyte growth/scatter factor (HGF/SF) is a crucial factor in normal tissue repair, leading to vascularization and re-epithelialization [1] via activation of the receptor tyrosine kinase MET [2]. The importance of MET for this process was demonstrated *in vitro* with MET-deficient mice keratinocytes that did not contribute to wound closure [1]. A lack of active HGF/SF was found in wounds with abnormal healing. This was caused by an increase in proteolytic enzymes, like plasma kallikrein and neutrophile elastase that degrade HGF/SF into antagonistic fragments [3]. Substitution of HGF/SF by administering a more stable MET receptor agonist, which is not a target of these enzymes, could be an option for therapeutic approach.

Internalin B (InlB), an invasion protein produced by *Listeria monocytogenes*, is a MET receptor agonist [4]. Intrinsically, InlB is a virulence factor and mediates the uptake of the bacterium into hepatocytes and various other cell types of the host organism [5]. InlB functionally mimics HGF/SF as shown in *in vitro* scatter assays with Madin-Darby canine kidney (MDCK) cells [4] and in proliferation assays with mouse keratinocytes [6]. Later, an InlB fragment spanning amino acids 36–321 ($InlB_{321}$) was shown to be sufficient for binding and activation of MET [7]. Although monomeric $InlB_{321}$ stimulates MET phosphorylation, it is not able to elicit cellular phenotypes like scattering or cell division [6]. Using structure-based protein engineering, $InlB_{321}$ could be turned into a full MET agonist by introducing two intermolecular disulfide bonds resulting in a dimeric protein with twofold (C_2) symmetry, referred to as $InlB_{321}$-crystal dimer ($InlB_{321}$-CD) [8].

Abbreviations: A549, adenocarcinomic human alveolar basal epithelial cells; s.d., standard deviation; HDF, human dermal fibroblasts; HGF/SF, hepatocyte growth factor/scatter factor; HaCaT, human adult keratinocytes, spontaneously immortalized under low calcium condition at elevated temperature; HEC gel, hydroxyethyl cellulose gel; $InlB_{321}$-CD, $InlB_{321}$ crystal dimer; mASC, mini artificial skin constructs; MDCK, Madin-Darby canine kidney; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PHK, primary human keratinocytes; Vero, Verda reno (kidney epithelial cell line extracted from an African green monkey).

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The biological potency of $InlB_{321}$-CD is similar to that of HGF/SF. In terms of stability and resistance to proteolysis, $InlB_{321}$-CD is superior to HGF/SF. In contrast to HGF/SF, $InlB_{321}$-CD is a non-glycosylated protein and large-scale production can easily be accomplished in *Escherichia coli* [9].

Aiming at topical administration on dermal wounds, an appropriate vehicle for protein drug delivery to skin is required. Hydrogels composed of hydroxyethyl cellulose (HEC) represent such a vehicle [10]. Additionally, moist wound environment from hydrogel administration accelerates re-epithelialization *in vivo* compared to dry conditions [11]. Finally it was shown that *in vitro* regeneration of sodium dodecyl sulfate-injured organotypic co-cultures was improved by hydrogels even without any therapeutic drug [12].

In this study, $InlB_{321}$-CD was investigated in terms of its potential to stimulate cutaneous wound healing. The work focuses on mitogenic and motogenic effects of $InlB_{321}$-CD on human keratinocytes, which have not been studied before in this context. As mainly epithelial cells express the MET receptor [13], an epithelial monolayer of HaCaT cells was chosen as a model system consisting of a non-tumoric immortalized cell line with an unchanged DNA fingerprint pattern during several passages [14]. An MTT assay was used to measure proliferation [15]. Migratory effects were analyzed in scratch assays [16] with or without arresting mitosis by prior incubation with mitomycin C, in order to distinguish migration from proliferation. Likewise, the migratory effect was further studied in a scratch assay with primary human keratinocytes to check whether immortalized HaCaT behaved differently from primary cell lines because of altered downstream signaling of the proto-oncogene MET.

Premature organotypic co-cultures of HaCaT cells and fibroblasts were used to study the influence of human fibroblasts, the natural source of endogenous HGF/SF in the skin [17]. Mature organotypic co-cultures were used to analyze negative effects of $InlB_{321}$-CD on a model of intact skin, e.g. promotion of invasive cell growth.

2. Materials and methods

2.1. HGF/SF, $InlB_{321}$-CD, $InlB_{321}$

Recombinant human HGF/SF was produced in CHO Lec3.2.8.1 cells following a published protocol [18]. The Cell-free supernatant was concentrated by ultrafiltration and then loaded onto a 25 ml heparin sepharose column that was preequilibrated with 250 mM Tris–HCl, pH 8.0, and 250 mM NaCl. After washing with 250 mM and 500 mM, the protein was eluted with 750 mM NaCl. HGF/SF was afterward activated with the specific protease HGFA while it was dialyzed against 4 l 200 mM Hepes, pH 7.4, and 250 mM NaCl overnight. HGF/SF was then loaded on a Mono S column and eluted with a linear NaCl gradient (250–1000 mM).

Monomeric and dimeric $InlB_{321}$ were produced according to a previous description [8]. $InlB_{321}$ and the G206C, A227C double mutant were expressed with N-terminal glutathione-S-transferase (GST) tag from vector pETM30 in *E. coli* BL21-CodonPlus (DE3) and purified by affinity chromatography on glutathione sepharose. The GST-tag was removed by cleavage with tobacco etch virus protease. Dimerization of the G206C, A227C double mutant was enhanced by dialysis against 20 mM Tris pH 8.0, 100 mM NaCl, 10 mM H_2O_2 to yield the homodimeric $InlB_{321}$-CD. Both the monomeric and the dimeric form were further purified via anion exchange chromatography and gel filtration. Purified proteins were analyzed by SDS–PAGE under reducing and non-reducing conditions and stored at −80 °C.

2.2. Human cell lines and co-cultures

Primary human dermal keratinocytes (PHK) (Invitrogen, Karlsruhe, Germany), were cultivated in a serum-free low calcium medium (K-SFM) supplemented with recombinant epidermal growth factor (rEGF, 0.2 ng/ml), bovine pituitary extract (30 μg/ml) (all Invitrogen, Karlsruhe, Germany) and gentamycin (5 μg/ml) (Biochrom, Berlin, Germany) until passage 6. They were subcultivated on collagen-I-coated plates (rat tail collagen-I 15 μg/ml in ethanol and acetic acid solution (1:1) according to reference [19]).

Immortalized human keratinocytes from the HaCaT-cell line [20] (a kind donation from N. Fusenig, DKFZ, Heidelberg, Germany) were cultivated in DMEM (Dulbeccos's Modified Eagle Medium) supplemented with 10 % (v/v) new born calf serum, 4 mM L-glutamine (all Biochrom, Berlin, Germany) and penicillin G (100 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 μg/ml) (all PAA, Pasching, Austria). They were used from passage 65–84.

Human dermal fibroblasts from the foreskin of newborns (HDF, Cascade Biologics, Mansfield, Great Britain) were cultured in DMEM according to standard conditions [21]. They were used from passage 3–12.

Organotypic co-cultures consisting of HDF and HaCaT cells (mASC) were manufactured as previously described with slight modifications [22]: Type-I collagen was extracted in acidic conditions from rat tails [19] in order to incorporate dermal fibroblasts for the dermal equivalent. The collagen dispersion was then cast into a 12-well-transwell with 3 μm pore size (Costar-Corning, Amsterdam, Netherlands) at a volume of 1 ml/well. Subsequent to gelation, the dermal equivalent was cultivated submerse in DMEM. One day later, 75,000 HaCaT cells were seeded onto each dermis and covered with medium. After one week of submerse cultivation, the constructs were lifted to the air–liquid-interface (ALI), DMEM was exchanged for two further weeks to MSBM 2% (minimally supplemented basal medium) which consisted of DMEM and Ham's F12 (Invitrogen, Karlsruhe, Germany; Biochrom, Berlin, Germany) (3:1) with supplementations reported previously [22].

Premature organotypic co-cultures were fabricated accordingly, but they were harvested 3 days before lifting them to ALI. Those with dead HDF were kept in the refrigerator at 4–8 °C for 3 days after casting the collagen dispersion and its gelation and prior to the subsequent treatment mentioned above.

2.3. Proliferation assay

HaCaT cells were seeded with a density of 60,000 cells/well in a 12-well plate (TPP, Trasadingen, Switzerland) and cultivated for three days. After washing with PBS, the medium was exchanged to serum-free DMEM. One day later, the keratinocytes were incubated for another day with 0.5, 0.05 and 0.01 nM $InlB_{321}$-CD versus equimolar concentrations of monomeric $InlB_{321}$ and medium. 30 nM HGF served as positive control. Afterward an MTT assay was carried out with modifications according to [22].

A hydroxyethyl cellulose gel (HEC gel) (Natrosol® 250 HX Pharm., Caelo, Hilden, Germany) was manufactured by distributing the gelling agent in phosphate buffered saline pH 7.4 (PBS) without calcium and magnesium (MP Biomedicals LLC, Illkirch, France) 0.6% (w/w). Afterward it was autoclaved and $InlB_{321}$-CD was added resulting in a concentration of 25 nM. This gel was then stored at 4–8 °C for 24 h or 7 days. These gels were tested versus the same concentration of $InlB_{321}$-CD in PBS to check for any incompatibilities of the protein with the gelling agent. Afterward, the buffered $InlB_{321}$-CD solution, the gel with the active compound as well as

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