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Development and application of three-dimensional skin equivalents for the investigation of percutaneous worm invasion

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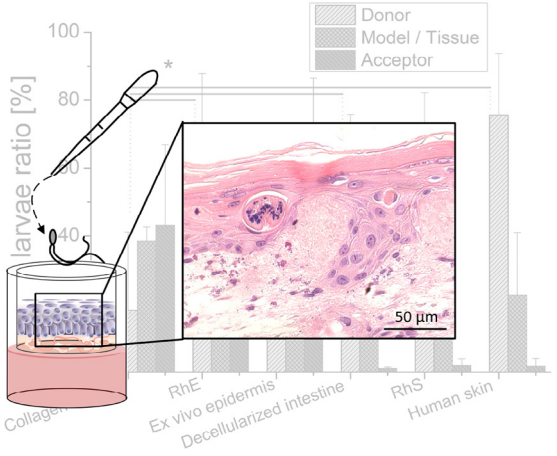
HIGHLIGHTS

- Skin equivalents accomplish the requirements for biological infection studies.
- Quantitative invasion assays demonstrate the capacity of skin equivalents.
- Skin equivalents provide a novel *in vitro* technology to analyze helminth invasion.
- Results support delayed tail loss hypothesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Investigation of percutaneous helminth infection is generally based on animal models or excised skin. As desirable replacement of animal experiments, tissue-engineered skin equivalents have recently been applied in microbial and viral *in vitro* infection models. In the present study, the applicability of tissue-engineered skin equivalents for the investigation of percutaneous helminth invasion was evaluated. Epidermal and a full-thickness skin equivalents that suit the requirements for helminth invasion studies were developed. Quantitative invasion assays were performed with the skin-invading larvae of the helminths *Strongyloides ratti* and *Schistosoma mansoni*. Both skin equivalents provided a physical barrier to larval invasion of the nematode *S. ratti*, while these larvae could invade and permeate a cell-free collagen scaffold and *ex vivo* epidermis. In contrast, the epidermal and full-thickness skin equivalents exhibited a human host-specific susceptibility to larvae of trematode *S. mansoni*, which could well penetrate. Invasion of *S. mansoni* in cell-free collagen scaffold was lowest for all experimental conditions.

Abbreviations: hDF, human dermal fibroblasts; hEK, human epidermal keratinocytes; RhE, reconstructed human epidermis; RhS, reconstructed human skin; *S. mansoni*, *Schistosoma mansoni*; *S. ratti*, *Strongyloides ratti*.

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Thus, reconstructed epidermis and full-thickness skin equivalents confirmed a high degree of accordance to native tissue. Additionally, not only tailless schistosomula but also cercariae could permeate the skin equivalents, and thus, delayed tail loss hypothesis was supported. The present study indicates that the limitations in predictive infection test systems for human-pathogenic invading helminths can be overcome by tissue-engineered *in vitro* skin equivalents allowing a substitution of the human skin for analysis of the interaction between parasites and their hosts' tissues. This novel tissue-engineered technology accomplishes the endeavor to save animal lives.

1. Introduction

A profound understanding of the percutaneous infection process of helminth larvae is of importance for the prevention and therapy of helminth diseases (Haas, 2003; McKerrow and Salter, 2002). Currently, larval invasion studies are based on animal models and excised animal skin (Franke et al., 2011; Stirewalt and Uy, 1969). In contrast to human skin samples, model organisms are characterized by a high availability. Additionally, the excision of animal skin can be standardized to specific topographical regions. Thus, compared to human skin samples from plastic surgeries, variations in thickness and state of the cornified layer are reduced in animal models (Takeuchi et al., 2011; Whitton and Everall, 1973). However, physiological and anatomical differences between human and animal skin limit the transferability of results from animal studies to humans (Evans et al., 2013; Godin and Touitou, 2007).

As an alternative to animal studies, *in-vitro*-generated organ-like skin equivalents have been developed (MacNeil, 2007; Persidis, 1999; Rheinwald and Green, 1977). These skin equivalents resemble the cellular and structural properties of native skin (Groeber et al., 2011). Hence, *in-vitro*-generated skin evolved to a standard tool for the characterization of pharmaceuticals, chemicals and cosmetics (Ponec, 2002). Skin equivalents provide high availability and can be generated with human cells. Advancing the concept of replacement, reduction and refinement of animal studies (3R's principle; Animal Ethics Infolink – Three Rs, 2014; Replacement, Refinement & Reduction of Animals in Research, 2014; Russell and Burch, 1959), tissue-engineered skin equivalents have recently been applied as microbial and viral *in vitro* infection models. Exemplarily, studies with *Candida albicans, Acinetobacter baumannii* and *Herpes simplex* have already shown the potential of skin equivalents for infection biology (Green et al., 2004; Hogk et al., 2013; MacNeil, 2007). There are only a few reports on the application of human skin equivalents investigating parasite–host interaction. Morgan and Arlian (2010) used a human skin equivalent to investigate the influence of cellular interactions between keratinocytes and fibroblasts when the cells were exposed to scabies mites, mite products, and mite extracts. Walochnik et al. (2009) adopted an organotypic skin equivalent for investigating the penetration of *Acanthamoeba* into the epidermis and the human tissue tolerability of miltefosine.

For the investigation of percutaneous helminth invasion, at least two characteristics of skin equivalents are required – the formation of a physical barrier to larval invasion and the host-specific stimulation of helminth invasion activity. Larval activation is determined by chemical factors that are synthesized and excreted by the skin (Chaisson and Hallem, 2012; Haas et al., 2002). The stimulation of parasites by these factors results in guidance to the host, target recognition and invasion through the skin barrier (Chaisson and Hallem, 2012; Dillman et al., 2012; Haas, 2003). The physical barrier of the skin is mainly generated by the *stratum corneum* and the cell–cell-junctions of the epidermis (Proksch et al., 2008). Larval penetration of the epithelial barrier is based on enzyme-mediated histolytic degradation as well as mechanical burrowing activity (Haas, 2003). Thus, in addition to skin-specific structural properties, an appropriate molecular composition is required to allow enzymatic degradation of the physical barrier. After penetration of the epidermal layer, the larvae are guided to the vasculature in the dermis via biochemical cues (Haas, 2003). The research group of Bartlett et al. (2000), Khammo et al. (2002) and Whitfield et al. (2003a) first applied Franz static one-chamber cell cultures and 3-dimensional skin equivalent and revealed a helminth infective larva in the dermis beneath the basement membrane within 10 min of exposure to infective larvae. However, detailed quantitative analysis of helminth penetration in skin equivalents of different complexity and applying two differing helminth species to test specific skin functions have not been performed yet, and thus, an ideal alternative to animal testing or excised human skin needs to be identified.

In the present study we examined the capacity of infective larvae from two most diverse helminths to penetrate or pass epidermal and complete skin equivalents as excised human epidermis and skin for control: *Strongyloides* (*phylum Nemathelminthes*) an intestinal nematode and *Schistosoma* (*phylum Platyhelminthes*) a vein-dwelling fluke.

First, the nematode *Strongyloides ratti*, genetically closely related to the human pathogenic *Strongyloides stercoralis*, penetrates the host through the skin (Haas, 2003; McKerrow and Salter, 2002; Sakura and Uga, 2010) the infective larvae secrete an collagenolytic metalloprotease like hookworms and filariae (Borchert et al., 2007; McKerrow et al., 1990) to facilitate the penetration, migrate from the subcutaneous infection site through muscular fibers, via the vascular circulation to the lungs and subsequently reside in the small intestine (Marra et al., 2011). *Strongyloides* exhibits fundamental differences from all other helminths: a direct, facultatively indirect development and autoinfection (Lok, 2007; Parasites – Strongyloides, 2012; Soblik et al., 2011). *Strongyloidiasis*, associated with cutaneous, gastrointestinal or pulmonary symptoms, is mainly endemic in tropical and subtropical regions, but has migrated from developing regions to industrialized areas (Parasites – Strongyloides, 2012).

Secondly, the infective larvae, cercariae, from the human-pathogenic trematode fluke *Schistosoma mansoni* were used in skin infection experiments. Cercariae emerging from infected intermediate snail host swim highly motile to reach the definitive human host by thermotaxis and chemotaxis, attracted by arginine, ceramide and fatty acids (Chaisson and Hallem, 2012; Haas, 2003; Haas et al., 2002). Invasion into the host skin is facilitated by elastase and metalloproteases degrading extracellular matrix proteins and cadherin (McKerrow and Salter, 2002). After entering the skin, the infective larvae transform into a tailless *schistosomula*, migrate via the circulation to its definitive portal or mesenteric vein habitat where they mature and produce up to thousands of eggs per day. *Schistosomiasis* (*Bilharziasis*) is the second most devastating parasitic disease of humans affecting the liver, spleen, bladder, kidney and intestine with more than 200 million people infected (Parasites – Schistosomiasis, 2014).

Especially the host-specificity of human-pathogenic helminths results in a lack of predictive infection models. For investigation of percutaneous larval invasion via *in-vitro*-generated skin equivalents, the reconstruction of structural and biochemical properties of human skin is essential. The aim of this study was to evaluate whether human skin equivalents provide an alternative to excised