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EXPERIMENTALLY INDUCED DISEASE

Histopathological and Immunohistochemical Studies of Cowpox Virus Replication in a Three-Dimensional Skin Model

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Summary

Human cowpox virus (CPXV) infections are rare, but can result in severe and sometimes fatal outcomes. The majority of recent cases were traced back to contacts with infected domestic cats or pet rats. The aim of the present study was to evaluate a three-dimensional (3D) skin model as a possible replacement for animal experiments. We monitored CPXV lesion formation, viral gene expression and cell cycle patterns after infection of 3D skin cultures with two CPXV strains of different pathogenic potential: a recent pet rat isolate (RatPox09) and the reference Brighton red strain. Infected 3D skin cultures exhibited histological alterations that were similar to those of mammal skin infections, but there were no differences in gene expression patterns and tissue damage between the two CPXV strains in the model system. In conclusion, 3D skin cultures reflect the development of pox lesions in the skin very well, but seem not to allow differentiation between more or less virulent virus strains, a distinction that is made possible by experimental infection in suitable animal models.

Keywords: three-dimensional skin culture; cowpox virus; gene expression

Introduction

Cowpox virus (CPXV), a member of the *Orthopoxvirus* (OPV) genus within the Poxviridae family, causes zoonotic infections and is endemic in Europe and Northern and Central Asia (Bennett and Baxby, 1996; Baxby and Bennett, 1997; Vorou *et al.*, 2008). The name cowpox was coined in the 18th century when CPXV was associated with clinical, but mostly localized, skin disease of cows and milkmaids. Today, human CPXV infections acquired from pet rats and cats are reported in increasing numbers (Vorou *et al.*, 2008; Campe *et al.*, 2009; Carletti *et al.*, 2009; Ninove *et al.*, 2009; Elsendoorn *et al.*, 2011; Vogel *et al.*, 2012; Hobi *et al.*, 2015), without evidence for direct human-to-human transmission. Field and experimental studies have indicated that CPXV maintains a reservoir in wild rodents such as bank voles (*Myodes glareolus*), field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*) or common voles (*Microtus arvalis*) (Bennett *et al.*, 1997; Feore *et al.*, 1997; Chantrey *et al.*, 1999; Hazel *et al.*, 2000; Burthe *et al.*, 2008; Begon *et al.*, 2009; Hoffmann *et al.*, 2015). Multiple divergent strains of CPXV have been identified; the best characterized are Brighton red (BR) and GRI-90 (Shchelkunov *et al.*,

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1998; Gubser *et al.*, 2004; Xu *et al.*, 2014). A number of full-length CPXV genomes are publicly available (www.poxvirus.org). It is also known that the pathogenic potential of different CPXV strains is variable. Infection of mice with BR results in general, but usually mild clinical signs on high titre inoculation (Martinez *et al.*, 2000). Infection of Wistar rats with CPXV strain BR also results in a mild to asymptomatic course of disease (Hoffmann *et al.*, 2015). In contrast, an isolate derived from a pet rat in Southern Germany in 2009, RatPox09, induces severe smallpox-like signs including rash in infected rats (Kalthoff *et al.*, 2011; Breithaupt *et al.*, 2012). Even in the case of intradermal inoculation, RatPox09 caused systemic disease with multiple lesions that range from proliferative to necrotizing and ulcerative, mixed cellular dermatitis and folliculitis with epithelial ballooning degeneration and necrosis as well as parakeratotic hyperkeratosis (Breithaupt *et al.*, 2012).

CPXV infection and disease have been reported in various captive mammals in zoological collections, where interspecies transmission was observed repeatedly and likewise with variable clinical signs, indicating different susceptibilities to infection that may also depend on the particular CPXV strain involved (Marennikova *et al.*, 1977; Baxby *et al.*, 1982; Pilaski *et al.*, 1986; Martina *et al.*, 2006; Kurth *et al.*, 2008; Kalthoff *et al.*, 2014).

Naturally occurring human infections are of growing concern because the number of people with vaccinia virus (VACV)-induced immunity is declining; even those that were vaccinated in childhood most probably no longer have a significant smallpox immunity (Shchelkunov, 2013). The normal course of infection in man was reconstructed from an overview of 54 cases (Baxby *et al.*, 1994). In contrast to smallpox, CPXV infection in man is due to direct inoculation of CPXV into compromised skin and/or oral mucous membranes via small lesions, after which the characteristic skin lesions develop. Firstly, inflamed macules arise that change to papulonodular or vesicular, then sterile pustules; later, central haemorrhagic necrosis covered by a black eschar and often surrounded by erythema and indurated oedema is formed (Baxby *et al.*, 1994). Fatal disease occurs rarely and is not completely characterized, although isolated cases of heart failure and encephalitis have been reported (Eis-Hubinger *et al.*, 1990).

Organotypic epithelial raft cultures are a promising system to recapitulate *in vitro* the structure, cell cycle status and physiological conditions of skin (Bell *et al.*, 1983); these are also currently being tested as a substitute for animal models (Roguet *et al.*, 2000). The Phenion® full-thickness skin model is a three-dimensional (3D) tissue construct that simulates histological and physiological properties of human skin (Dorn *et al.*, 2006; Mewes *et al.*, 2007; Ackermann *et al.*, 2010). The model represents a multilayered epidermis and a dermal compartment, where the epidermis is generated from male neonatal foreskin keratinocyte stem cells and the dermis from fibroblasts embedded in a bovine collagen matrix. Keratinocytes and fibroblasts are obtained from biopsy material of the same healthy human donor (www.phenion.com). The pathogenesis of several different virus infections, including infections with papillomaviruses, adenoviruses, parvoviruses, poxviruses and herpesviruses, have been tested with 3D skin substitutes (Andrei *et al.*, 2010). In the case of poxviruses, they were used to test the pharmacokinetics of antiviral drugs for CPVX and VACV infections (Duraffour et al., 2007; Snoeck *et al.*, 2002).

We tested the suitability of a 3D skin model to assess CPXV replication and virulence prediction by examining the gene expression patterns, tissue damage, cell cycle status and regulation in the Phenion® full-thickness skin model infected with two different CPXV strains. We used two CPXV strains, BR and RatPox09, which show very different virulence and pathology *in vivo*. Our results indicate that, while CPXV skin replication can be successfully modelled using this in-vitro system, an assessment of overall virulence was not possible, likely because important steps in the pathogenesis, such as development of viraemia and innate or non-specific immune response, are absent.

Materials and Methods

Viruses

CPXV strain BR (AF428758) was kindly provided by Dr. P. Beard, University of Edinburgh, UK, and used as reference strain. CPXV strain RatPox09 was isolated from a diseased pet rat, which had infected two people in Southern Germany in 2009 and was kindly provided by Dr. H. Meyer, Munich, Germany (Kalthoff *et al.*, 2011). Both strains were propagated on Vero76 cells and amplified to stock titres of approximately 10^7 $TCID_{50}$/ml.

3D Skin Model Infections

The Phenion® full-thickness skin model Phenion®FT, obtained at the age of 14 days at air–liquid interphase (Ø 1.32 cm; surface area 1.30 cm^2), was purchased from Henkel AG & Co. KGaA (Düsseldorf, Germany). According to instructions of the supplier, the tissue was kept at 37°C under 5% CO_2 overnight and fresh, pre-warmed medium was added.