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Review

Epithelial raft cultures for investigations of virus growth, pathogenesis and efficacy of antiviral agents

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ABSTRACT

The organotypic epithelial raft cultures, originally developed to study keratinocytes differentiation, represent a novel approach to the study of viruses able to infect epithelial cells. Organotypic epithelial raft cultures accurately reproduce the process of epithelial differentiation *in vitro* and can be prepared from normal keratinocytes, explanted epithelial tissue, or established cell lines. This culture system permits cells to proliferate and fully differentiate at the air–liquid interface on a dermal-equivalent support. Normal primary human keratinocytes (PHKs) stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, while transformed cell lines exhibit dysplastic morphologies similar to the (pre)neoplastic lesions seen *in vivo*. This three-dimensional (3D) culture system provides an essential tool for investigations of virus growth, virus–host cell interactions, for the genetic analysis of viral proteins and regulatory sequences, and for the evaluation of antiviral agents. The 3D epithelial cultures have proven a breakthrough in the research on papillomaviruses, since their life cycle is strictly linked to the differentiation of the host epithelium. In the last years, several reports have shown the usefulness of the 3D epithelial cultures for the study of other viruses that target at least during a part of their life cycles epithelial cells. The 3D epithelial cultures allow the analysis of virus–host cell interactions in stratified epithelia that more closely resemble the *in vivo* situation. In this review we describe the advances on research on 3D epithelial cultures for the study of virus growth and pathogenesis of different families of viruses, including papilloma-, herpes-, pox-, adeno-, and parvoviruses.

Contents

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1. Introduction to epithelial raft cultures

Numerous attempts have been performed by investigators to accurately culture three-dimensional (3D) epithelium *in vitro*. These efforts have led to the development of organotypic cultures that accurately mimic the *in vivo* physiology of the epidermis (Asselineau and Prunieras, 1984). The organotypic epithelial raft culture model, originally developed to study keratinocytes differentiation, has been very useful for the investigation of virus replication in a differentiated epithelium. This represents a novel approach to the study of human viruses able to infect epithelial cells. Pathogenic viruses use human epithelium as the initial site of infection, the site of replication, and a site for latency or persistence (Bodily et al., 2006). The organotypic epithelial raft culture system reproduces the authentic program of differentiation, providing an essential tool for investigations of virus growth, pathogenesis and for the genetic analysis of viral proteins and regulatory sequences. Organotypic epithelial raft cultures accurately reproduce the process of epithelial differentiation *in vitro*. When undifferentiated confluent keratinocytes monolayers are grown at the air–liquid interface, the cells become polarized, and form tight junctions prior to the initiation of epithelial differentiation. The keratinocytes remaining in the basal layer maintain their proliferative potential, while a part of the cells leave the basal layer, lose mitotic capacity, and differentiate into mature epithelial cells, forming an epithelium with the characteristic multilayered structure. Keratinocytes follow a programmed pattern of gene expression during differentiation, each step being characterized by the expression of keratin and keratin-associated proteins. This represents a unique tool for the study of viruses that depend on epithelial differentiation for their growth (Andrei, 2006). The 3D epithelial cultures have proven a breakthrough in the research on human papillomaviruses (HPV), since their life cycle is strictly linked to the differentiation of the host epithelium. In the last years, several reports have shown the usefulness of the 3D epithelial cultures for the study of other viruses that target epithelial cells, at least during a part of their life cycles. In this review we describe the advances on research on 3D epithelial cultures for the study of virus growth and pathogenesis.

2. Preparation of epithelial raft cultures

2.1. From dispersed primary keratinocytes

Most laboratories use an adaptation of a technique described by Asselineau and Prunieras (1984) for the preparation of organotypic epithelial raft cultures. Fibroblasts are mixed together with type I rat tail collagen reconstituted with media and buffer at 0 °C and then placed in tissue culture dishes and incubated at 37 °C until the collagen mixture solidifies (Fig. 1A). This collagen matrix forms a lattice or raft that will act as the dermal equivalent. Murine fibroblasts such as Swiss 3T3 J2 or Balb/C 3T3 JCA 31 that do not divide rapidly or have much propensity to migrate up to the dermal surface are recommended. After equilibration of the collagen/fibroblasts matrix with medium, the epithelial cells of choice are seeded on top of the matrix, and allowed to grow to confluence while remaining submerged. The rafts are then lifted and placed on the top of stain-less grids and incubated at the air–liquid interface. The epithelial cells are nourished by a medium containing growth factors, serum, and a high concentration of calcium via capillarity action through the dermal equivalent. The incubation time is determined empirically and is dependent on the type of epithelial cells used. If primary human keratinocytes are used to prepare the rafts, during the incubation time the cells stratify and differentiate into a full-thickness tissue mimicking the *in vivo* situation (Fig. 1A). A well organize epithelium complete within 8–10 days. Such cultures can be maintained for 3–5 weeks, although the epithelium becomes thinner with time because cell proliferation within the basal layer gradually diminishes and programmed cell death in the uppermost strata increases. Once the rafts have stratified they can be harvested and examined by standard histological, molecular, and biochemical techniques.

2.2. From explanted epithelial tissue

A part of normal or diseased epithelial tissue biopsies can be applied directly onto the dermal equivalent and grown at the air–liquid interface (Fig. 1B). Epithelial tissues from different parts of the body, including foreskin, larynx, and cervix can be used in the raft culture model. The fibroblasts in the dermal component of surgical specimens remain in the biopsy chips, while the epithelial cells that maintain the ability to divide (those within the basal and parabasal layers) migrate across the dermal equivalent. Such outgrowth appears to be analogous to the healing from wound margins.

2.3. From established cell lines

Raft cultures can also be prepared using cells derived from neoplasias of all grades and in this case the epithelium that emerges has dyspalstic morphology, incomplete differentiation, and abnormal histology (Fig. 1A). Therefore raft cultures represent also a powerful tool for the study of epithelial neoplasia and tumorigenesis. The raft culture technique has been used to grow human papillomavirus (HPV)-immortalized primary human keratinocytes (PHKs) as well as cervical carcinoma cell lines harboring HPV, reproducing in this case the histopathology observed in cervical carcinomas *in vivo*. Some, but not all, squamous cell carcinoma (SCC) lines that do not harbor HPV DNA sequences also maintain the ability to stratify into dysplastic epithelium.

3. Viruses with tropism for epithelial cells

As cutaneous or mucosal epithelia are the site of replication of several viruses, characterization of their replicative cycles in organotypic epithelial raft cultures represent a relevant model for studying virus–host cell interactions and the effects of antiviral and/or antitumor agents. In some cases, complete viral replication is restricted to epithelial cells (i.e. papillomaviruses), while in most of the cases, epithelial cells are one of the cell types infected by viruses.

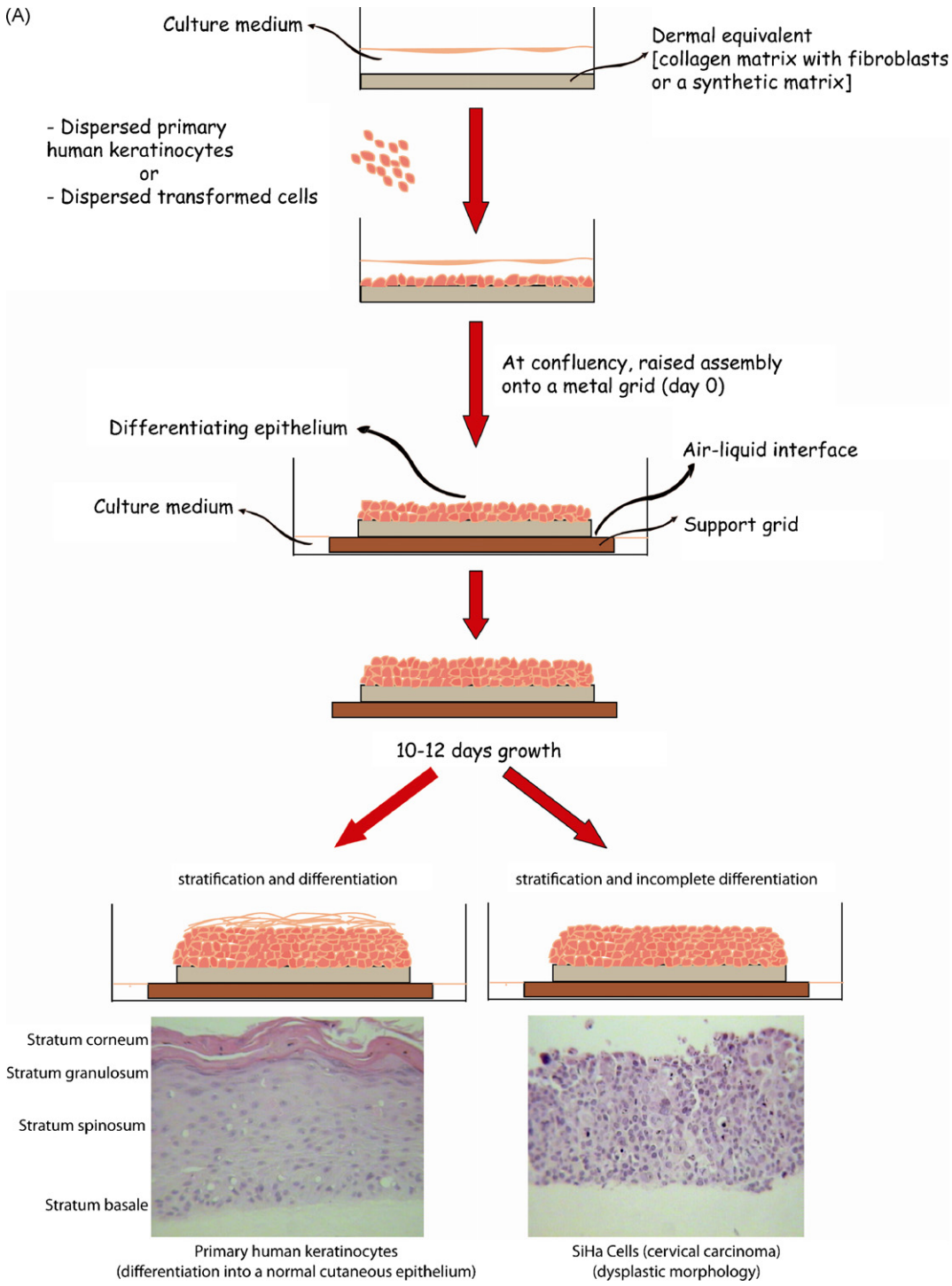

Fig. 1. Preparation of organotypic epithelial raft cultures from dispersed cells (A) or tissue biopsy explants (B). The dermal equivalent is a matrix composed of fibroblasts embedded into rat tail type 1 collagen. (A) Dispersed primary keratinocytes, for example isolated from neonatal foreskins or cell lines are seeded on top of the collagen matrix in a 24-well tissue culture plate and allow growing under the liquid medium for 24–48 h till they reach confluence. The dermal equivalent is loosened from the cell wall, and the assembly is placed onto stainless grids and transferred to 60 mm dishes, with the bottom of the collagen in contact with the culture medium and the upper surface with the epithelial cells in contact with the atmosphere. The confluent normal keratinocytes stratify and differentiate in 10–12 days. When transformed cell lines are used, the cells stratify but they do not differentiate and produce a multi-layered epithelium with dysplastic morphology and abnormal histology. The cultures can be maintained for a couple of weeks more until the keratinocytes lose their proliferative potential or when the feeder cells in the collagen matrix die. (B) Whole tissue biopsy explants (2–3 mm^2) are placed directly on top of the collagen matrix and immediately maintained at the air–liquid interface. Keratinocytes with proliferative capacity grow out from the explants to the collagen matrix and differentiate while the fibroblasts present in the explants remain at the place and do not grow out from the explants.
Adapted from Chow and Broker (1997).

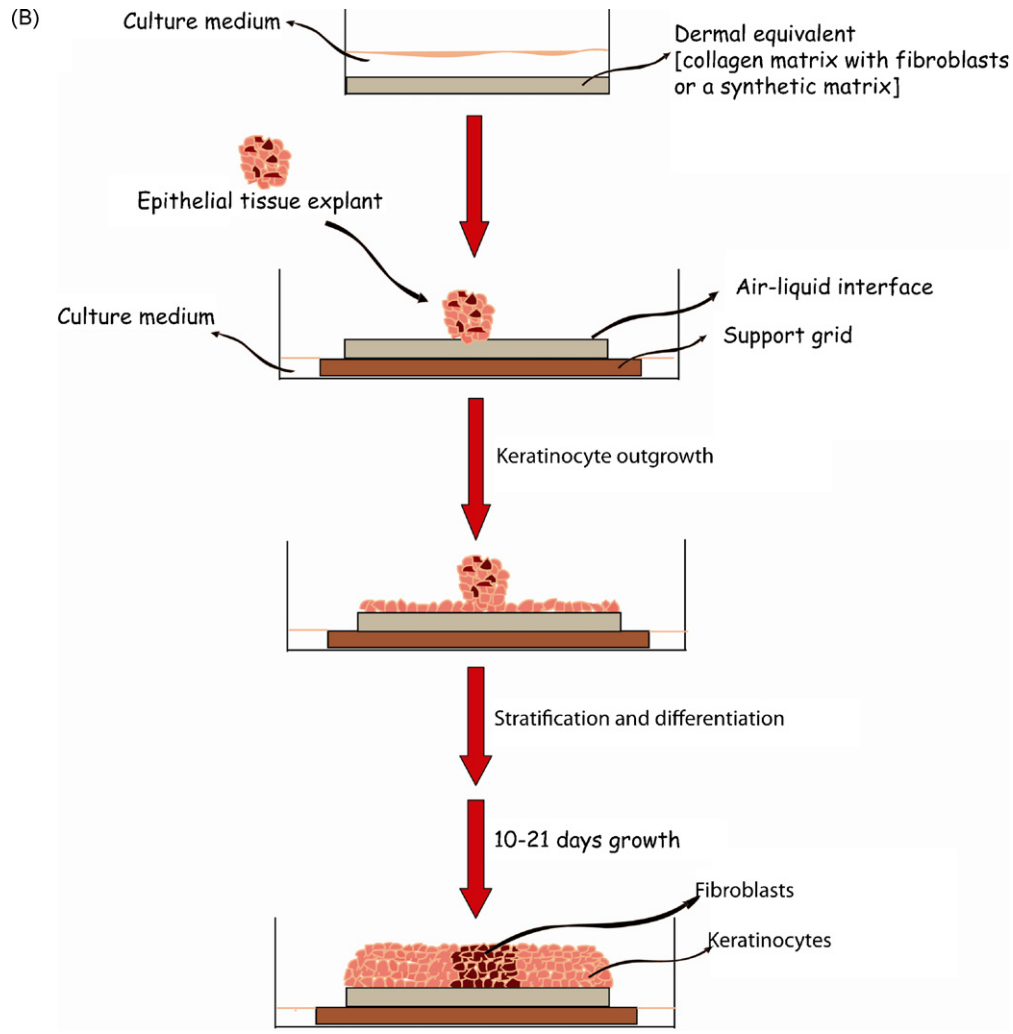

Fig. 1. (*Continued*).

4. Papillomaviruses

Papillomavirus are small DNA viruses with a strict epithelial tropism, infecting keratinocytes of the stratified cutaneous or mucosal surfaces where they induce a range of host responses (Doorbar, 2005; Stanley, 2006). Over 120 viral genotypes of human papillomaviruses (HPV) have been now described and they can be categorized on the basis of site of occurrence (mucosal or cutaneous), but also in high and low risk groups in function of their potential to induce the development of malignant proliferation (de Villiers et al., 2004). Although the majority of infections are associated with benign proliferation, some HPVs are associated with intraepithelial neoplasia (cervical, vaginal, vulvar or penile) and epidermodysplasia verruciformis (Bosch et al., 2002; Castellsague, 2008; Parkin and Bray, 2006). Despite the strong epidemiological association between infection with high-risk HPV types and the development of anogenital cancer, only a subset of patients infected with high-risk HPV types progress to invasive squamous carcinoma. Most infections are self-limiting and ultimately are cleared by the host, but in a small number of patients persistent lesions can progress to cancer. The ability of HPV infections to progress to malignancies has been attributed to the actions of the E6 and E7 proteins and their ability to modify cell cycle regulators (Howie et al., 2009; McLaughlin-Drubin and Munger, 2009; Munger et al., 2004). One important function of high-risk E6 proteins is the binding of the tumor suppressor p53, which results in its ubiquitin-mediated degradation. The primary activity of high-risk E7 proteins is its association with members of the retinoblastoma (Rb) tumor repressor family to facilitate progression to the S phase. Progression to malignancy is characterized by the expression of E6 and E7 proteins in dividing cells, chromosomal instability, and progressive ability to resist both innate and adaptive antiviral immune defenses (Fehrmann and Laimins, 2003; Longworth and Laimins, 2004). Integration of HPV genome into the host chromosome is seen in most invasive cancers and high grade lesions. It may be an early event in cancer progression and can occur randomly throughout the genome, often resulting in the loss of E2 and deregulation of E6/E7. However, there seems to be preferential integration near common fragile sites (CFS), specific chromosomal loci that are particularly prone to forming double-strand breaks (Dall et al., 2008; Wentzensen et al., 2004).

HPVs virions infect the epithelium through micro-lesions that allow the virus to get in contact and infect the basal cells (Doorbar, 2005). Following infection, the virus migrates then to the nucleus and is maintained in as an episome at approximately 50–100 copies per cell. In these infected basal cells, episomal HPV DNA is replicated in a coordinated way with cellular DNA and following cell division, viral episomes are equally distributed to each daughter cell. Once that cell division is accomplished, one of the infected daughter cells get loose from the basal membrane and migrates towards the suprabasal layers, and starts to differentiate. The infected cells that remain in the basal layer allow the persis-

tence of the virus. Suprabasal cells normally exit the cell cycle and begin the process of terminal differentiation but HPV stimulates cell proliferation. The increased proliferation of suprabasal epithelial cells is attributed to the expression of the viral oncoproteins E6 and E7. Viral DNA amplification to several thousand copies per cell occurs only upon differentiation of cells in the suprabasal layer. HPV late-gene expression occurs in the granular layer following DNA amplification, resulting in the assembly and subsequent release of progeny virions in the upper layer of the epithelium. One of the challenges in studying the productive life cycle of HPV has been the difficulty in reproducing the process of cell differentiation *in vitro*.

4.1. Epithelial raft cultures for the study of papillomaviruses

The link of papillomavirus replication cycle to differentiation of the host epithelial cells has hampered the study of HPV for many years. The development of organotypic raft cultures that reproducibly form a squamous epithelium that closely resembles the original tissue fulfills the essential requirement for investigating different aspects of HPV life cycle and this culture system has proven to be a step forward on HPV research (McLaughlin-Drubin et al., 2003, 2005; Meyers et al., 1992). HPV-positive cells are either derived from biopsies or created by the efficient introduction of HPV sequences (via recombinant retroviruses or lipofection to keratinocytes) (Chow and Broker, 1997). Organotypic epithelial raft cultures of HPV-immortalized cells, reproducing different grades of neoplasia, allowed to perform extensive and deep analysis of the functions of viral regulatory and coding sequences and to examine virus–host interactions (Table 1). Importantly, this system permitted the production of infectious HPV virions for the first time (Dollard et al., 1992; Meyers et al., 1992), and several researchers have further used the organotypic epithelial raft cultures to study HPV biology (Fang et al., 2006; Flores et al., 1999; Garner-Hamrick et al., 2004; Lambert et al., 2005; Meyers et al., 1997, 2003; Wilson et al., 2005). Recently, a highly efficient and reproducible system that generates autonomous HPV-18 genomes in primary human keratinocytes in the organotypic raft culture system has been described (Chow et al., 2009; Wang et al., 2009).

A detailed review of the use of organotypic epithelial raft cultures for the study of the natural history of HPV has been recently performed by Bodily et al. (2006). In the present overview we will focus on data concerning the use of epithelial raft cultures for evaluating the efficacy of therapeutic interventions for HPV. So far, these studies appear to be rather limited, mostly due to the difficulty in setting an endpoint for activity and in providing a correct measurement of selectivity of the agents. Most of the reports are based on morphological alterations of the rafts observed following treatment with the different agents without quantification of the antiviral effects. Studies using either monolayer cultures or raft cultures to determine the efficacy of interferon (INF) or retinoic acid therapy for the treatment of cervical cancer have not provided conclusive results. Retinoic acid-treated rafts of HPV16-immortalized endocervical cells (HEN-16 and HEN-16-2 cells) formed epithelia composed of two to three cell layers of columnar-like cells resembling simple epithelium of the endocervix in contrast to untreated rafts that showed dysplastic morphology similar to carcinoma in situ observed in women (Shindoh et al., 1995). Electron microscopy and cytokeratin expression patterns confirmed the histology of a differentiated endocervical phenotype following treatment with retinoic acid. However, expression of HPV16 E7 was modestly lower in treated epithelia. In contrast, when two tumorigenic cells [i.e. HEN-16T and HEN-16-2 derived, respectively, from HEN-16 and HEN-16-2 cells] were tested, HEN-16T cells were significantly less sensitive than the normal and immortalized non-tumorigenic cells, and the HEN-16-2T cells were completely resistant (Sarma et al., 1996). Moreover, the rafts from both tumorigenic cell lines were resistant to retinoic acid and showed dysplastic morphology.

Table 1
Papillomaviruses and organotypic epithelial raft cultures for the study given below.

A. Differentiation-dependent molecular biology of the virus life cycle
Viral protein functions
Viral promoters and transcriptional regulation of viral genes
Cis elements and mechanisms of regulation
Viral DNA replication
Late protein expression
B. Production of infectious virus
C. Virus–host interactions
Reactivation of host DNA synthesis in differentiating cells
Deregulation of cell cycle proteins by viral oncoproteins (high-risk versus low-risk HPVs)
D. Neoplastic progression of HPV associated lesions
Function of viral oncogenes
Chromosomal abnormalities
Host specific histological changes accompanying progression
Deregulation of specific host proteins [e.g. gap junction proteins, integrins, and c-myc protoncogene]
Viral integration
E. Loss of host tissue differentiation following HPV infection and transformation
Viral oncogenes and their effects on the host tissue differentiation and morphology
Host proteins as markers of differentiation (i.e. specific keratins, cell cycle markers such as PCNA, and cell cycle regulatory proteins such as p53)
Loss of cellular response to growth factors signaling pathways (i.e. TGF-β1)
F. Cofactors necessary for progression to malignancy
Cigarette smoking
Steroid hormones
Co-pathogens such as HSV
G. Interaction of HPV with other epitheliotropic viruses
Adeno-associated virus type 2 (AAV2)
HSV
Adenoviruses
H. Immunobiology of HPV
Interaction of infected keratinocytes with cells and soluble factors of the immune system
I. Therapeutic testing

Massad et al. (1996) compared the effects of INF-α and all-*trans*-retinoic acid on proliferation of several cell lines derived from cervical cancers. Exposure to pharmacological concentrations of either INF-α or all-*trans*-retinoic acid for 72 h inhibited growth of cervical cancer lines ME-180, 283, SiHa, C33A, 621, CaSki, HeLa, and B132. However, CaSki and SiHa cells exposed to INF-α or all-*trans*-retinoic acid or both for 9 days developed resistance to growth inhibition, and growth resumed at a rate comparable to control after removal of the agents. In organotypic cultures, cells showed morphology similar to carcinoma in situ, and exposure to INF-α and all-*trans*-retinoic acid for 14 days did not affect this morphology. Furthermore, no changes in markers of differentiation were noted following immunohistochemical analysis of cytokeratin and involucrin expression. In a later study, cell growth inhibition by treatment with retinoic acid, INF-α, and their combination was demonstrated to differentially depend on treatment type, cell origin, cell line, and oncogenic status (Yokoyama et al., 2001). In this study, retinoic acid was shown to reduce dysplastic differentiation in raft cultures of HPV18-immortalized cells and IFN-α induced apoptosis.

The inhibition of growth of normal and HPV-transformed keratinocytes in monolayer and organotypic cultures by INF-γ and tumor necrosis factor-α (TNF-α) has also been reported (Delvenne et al., 1995). INF-γ reduced the DNA synthesis of normal keratinocytes and HPV-transformed keratinocytes in monolayer cultures (as measured by [^{3}H]TdR incorporation and fluorimetric

DNA titration) and afforded a marked growth inhibitory effect in organotypic raft cultures (as measured by the ability of the cells to stratify, and by determination of Ki67 antigen). In control raft cultures, normal keratinocytes produced an epithelial sheet of about 10 layers that closely resembled normal cervical epithelium, and was characterized by sparse Ki67 antigen-positive cells; whereas HPV-transformed keratinocytes produced up to 15 poorly differentiated epithelial layers comparable to high grade cervical lesions. When normal and HPV-transformed rafts were maintained in the presence of INF-γ, the epithelial sheet was reduced to a few cells in thickness and the density of Ki67 antigen-positive cells was decreased. The combination of INF-γ and TNF-α resulted in a more pronounced cell growth inhibitory effect in monolayer and organotypic cultures. TNF-α alone reduced the DNA synthesis of normal keratinocytes but was significantly less active than INF-γ to inhibit the growth of HPV-transformed keratinocytes.

Sen et al. (2005) tested the effects of two commercially available IFNs, interferon-α-n3 (Alferon N) and interferon-α-2b (Intron A), on HPV vegetative replication in raft cultures able to sustain the complete viral life cycle of HPV 16, 18, and 31b. These studies showed that the two IFNs differed in their ability to affect viral load of the different HPV types. Treatment with an increasing concentration of IFN-α preparations did not always correlate with a stepwise inhibition of HPV replication. This is in keeping with the inconsistent results obtained using monolayer cultures to determine the efficacy of INF therapy for the treatment of cervical cancer.

Transforming growth factor β1 (TGF-β1) is a potent growth inhibitor for a variety of cultured cells, including many normal human epithelial cells. Although most normal epithelial cells were shown to be responsive to TGF-β1, many transformed cell lines proved resistant to its effects, suggesting that loss of responsiveness to growth-inhibition by TGF-β1 may be an important step in tumorigenesis. There have been conflicting reports on the ability of TGF-β1 to inhibit the growth of HPV+ keratinocytes in monolayer cultures (Dagnino et al., 1993; Sporn and Roberts, 1990) and regulation of HPV gene expression (i.e. down-regulation of HPV E6 and E7 gene transcription) was proposed as the mechanism by which TGF-β1 might exert growth-inhibitory effects on HPV-infected cells (Braun et al., 1992; Woodworth et al., 1990). Ozbun and Meyers (Ozbun and Meyers, 1997) employed the organotypic epithelial raft tissue culture system to investigate the TGF-β1 response of HPV-positive keratinocytes derived from neoplastic biopsies. Growth of these cell lines as raft tissues showed that many of them were altered in the ability to stratify and synthesize differentiation-specific proteins whereas addition of TGF-β1 to the raft tissues resulted in a more complete differentiation similar to treatment with 12-O-tetradecanoyphorbol-13-acetate (Ozbun and Meyers, 1997). Interestingly, TGF-β1 treatment of HPV+ raft epithelia led to a dose-dependent increase in E7 RNA expression in contrast to results from previous studies with monolayer cultures.

Based on the fact that expression of EGF receptor (EGFR) was abundant throughout the thickness of the epithelium of HPV-positive rafts and it was less expressed only in the basal layer of rafts of normal cervical keratinocytes, Renard et al. (2002) tested bispecific antibodies, which bind both the EGFR and CD3 in the raft culture model. These antibodies facilitated the infiltration of T cells into carcinoma rafts and increased killing of the HPV-positive cells by the lymphocytes, while they had no effect on T cell infiltration into rafts of normal keratinocytes. On the contrary, Mason et al. (1999) could not demonstrate an effect of vitamin E in the raft culture model since little difference between vitamin-treated and control cultures, except for a reduction in the number of layers in the vitamin-treated cultures, were observed.

The major tea polyphenol, (−)-epigallocatechin (EGCG) was shown to decrease the thickness of rafts of both HPV-immortalized and HPV-transformed cells and to inhibit telomerase activity regardless of the cell type used (Yokoyama et al., 2004). However, induction of apoptosis following EGCG treatment was detected in raft cultures grown from HPV18 immortalized cells but not from HPV18 transformed cells. The adverse effects of microbicides (i.e. nonoxinol 9, benzalkonium chloride, chlorhexidine and cholic acid) have also been evaluated in the raft culture model (Aebischer and McDougall, 1997). Following repeated treatment of rafts from normal keratinocytes and HPV-immortalized cells, harmful effects (histological alterations) were seen, while no alterations were observed in undifferentiated monolayer culture models.

The acyclic nucleoside phosphonate (ANP) analogues display a broad spectrum of activity against a range of DNA viruses and retroviruses. In addition to their antiviral activity, several of the ANPs exhibit cytotoxicity towards proliferating cells, including HPV-positive cells. The selectivity of PMEG [9-(phosphonylmethxyethyl)guanine] and its prodrug cPr-PMEDAP [9-(2-phosphonylmethoxyethyl)-N6-cyclopropyl-2,6-diaminopurine] compared to cidofovir (CDV, HPMPC) against HPV has been evaluated in organotypic epithelial raft co-cultures of primary human keratinocytes isolated from neonatal foreskins and the cervical carcinoma cell line SiHa (HPV-16 positive) (Fig. 2A) (Andrei et al., 2009b). In control untreated co-cultures, rafts showed regions with dysplastic morphology, normal epithelium and areas with mixtures of both types. In contrasts, rafts that were treated with PMEG, cPr-PMEDAP and CDV showed areas of fully differentiated normal epithelium and destruction of the tumor cells (Fig. 2B). Inhibition of SiHa cell proliferation in the rafts by the different ANPs was concentration- and time-dependent, and quantification of the area and the number of both tumor and normal cells was performed by image analysis. When rafts were allowed to differentiate for 3 days and medium containing serial dilutions of the compounds were added for 7 subsequent days till the cultures were fixed, 50% of tumor cells were quantified in untreated control cultures. Rafts treated with PMEG at concentrations of 5, 2, 0.5 and 0.2 μg/ml had, respectively, 0, 15, 37, and 26.1% of tumor cells while those treated with cPr-PMEDAP presented, respectively, 8, 10, 21, and 40% of tumor cells. Doses of 20, 5 and 2 μg/ml of CDV decreased the percentage of tumor cells to, respectively, 1, 6, and 47%.

5. Herpesviruses

All human herpesviruses can infect epithelial cells. For some human herpesviruses, a clear tropism for epithelial cells is a characteristic feature; other members of the Herpesviridae have an obvious tropism for other cell types although epithelial cells can be infected under certain conditions.

5.1. α-Herpesviruses

Human herpesviruses 1, 2, and 3 [herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) and varicella-zoster virus (VZV), respectively], have been classified as α-herpesviruses and have an ability to establish latent infections in sensory ganglia. They can also infect epithelial cells of mucous membranes and skin. These epithelial sites provide points of production of infectious virus that can be transmitted to other individuals. HSV is one of the most ubiquitous human viruses. HSV-1 and HSV-2 infections are among the most common infections worldwide, although seroprevalence varies widely by country, region within individual countries, and population subgroup (Nahmias et al., 1990; Smith and Robinson, 2002; Tran et al., 2004). HSV is typically responsible for mucocutaneous lesions of the mouth and genital organs in humans. HSV-1 is most commonly associated with orofacial herpes, whereas HSV-2 primarily causes anogenital herpes. Following replication at the primary site of infection, HSV enters

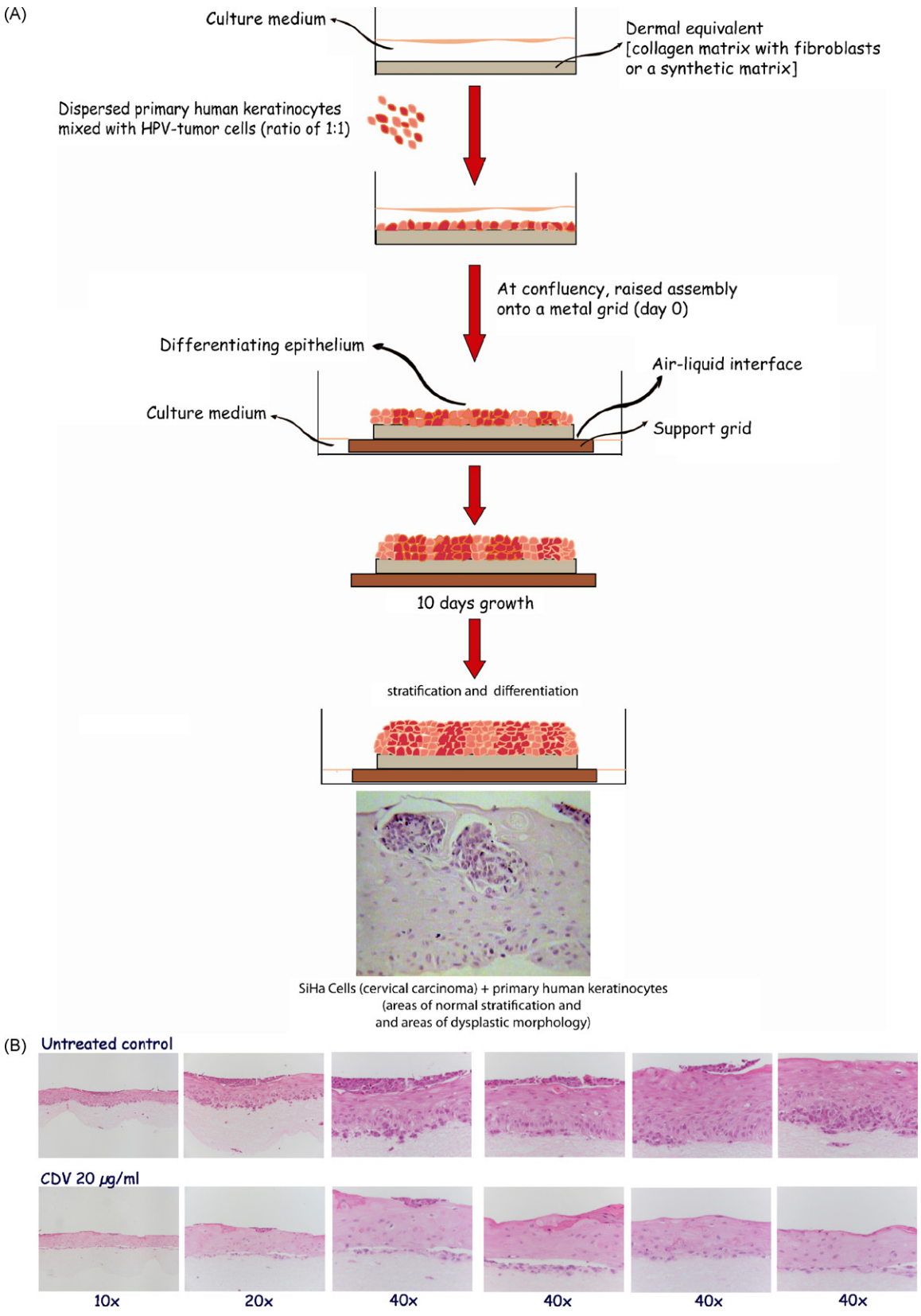

Fig. 2. Co-cultures of primary human keratinocytes and HPV-positive cells to evaluate the selectivity of anti-HPV agents. (A) Normal and tumor cells are mixed at a ratio of 1:1 and seeded on top of the collagen matrix in a 24-well tissue culture plate. After 24 h of growth under liquid medium, the dermal equivalent is lifted and placed onto stainless support grids in 60 mm dishes. At different stages post-differentiation at the air–liquid interface, compounds can be added either to the culture medium or on top of the rafts. In control untreated co-cultures, tumor cells and normal keratinocytes grow in two separate groups. (B) Hematoxilyn–eosin staining of rafts prepared with co-cultures of normal keratinocytes and HPV-16 positive cells (SiHa cells) treated or not with cidofovir (CDV). At day 3 post-lifting of the rafts, different concentrations of the compound were added to the culture media and the raft cultures were allowed to proliferate for 7 more days. After a total of 10 days of growth the rafts were fixed and processed for histological analysis.

local nerve endings and by axonal transport the virus reaches the dorsal root ganglia, where it establishes and maintains lifelong latent infections. Periodically, the virus reactivates, multiplies, and is transported through the axon back to a portal of entry, where it gives characteristic skin lesions.

Primary infection with VZV results in chickenpox (varicella), a common childhood illness. Chickenpox is highly contagious and is easily spread from person to person in airborne respiratory droplets from an infected person's coughing or sneezing or with the fluid from open sores. Similar to all herpesviruses, VZV establishes a latent state following the primary infection. VZV remains latent in the posterior root ganglia, and reactivation of the virus results in skin lesions characteristic of herpes zoster (shingles), which manifests as a localized rash in a unilateral, dermatomal distribution that is often associated with severe neuropathic pain, called postherpetic neuralgia (PHN).

HSV and VZV are responsible for typical skin lesions presenting characteristic histological features. The histological characteristics of HSV and VZV lesions are well known and have been carefully described in the past. For example, changes induced by HSV infection of the skin include ballooning of infected cells followed by subsequent degeneration of the cellular nuclei, generally within parabasal and intermediate cells of the epithelium. The cells loose intact plasma membranes and evolve to form multinucleated giant cells. Upon cell lysis, clear fluid containing large quantities of virus accumulate between the epidermis and dermal layer and form vesicles (Whitley, 1993). Characteristic histological changes of VZV infection include balloon degeneration of infected cells, with the formation of intranuclear inclusions and multinuclear giant cells. Infected cells have marginated chromatin. Early in the course of infection, the nuclei may contain homogeneous basophilic material, but more often they showed a rounded eosinophilic body surrounded by a wide clear zone. Multinucleated giant cells may contain up to 30 nuclei, each with an inclusion body (Gelb, 1993). Similar cells were seen in herpes zoster and HSV infections, but not in vesicular lesions produced by pox or enteroviruses. Considering that keratinocytes are the main target cells for productive infection *in vivo* for both HSV and VZV, the 3D raft culture system represents a relevant model for studying the course of HSV and VZV infections in a differentiated epithelium *in vitro*.

5.1.1. Investigations of Herpes simplex virus (HSV) growth in 3D epithelial raft cultures

Syrjanen et al. (1996) were the first to report the applicability of the organotypic 3D raft culture system for the study of herpes simplex virus (HSV). They described the establishment of lytic and non-productive infection by HSV-1 (F strain) in squamous epithelial cells cultures in a 3D organotypic tissue culture. For the tissue culture, HaCaT cells (immortalized skin keratinocytes) and normal fibroblasts (feeder cells) derived from the skin were used. The cultures were infected with 5 PFU either when the epithelial cells had grown to about 80% confluent on the collagen fibroblast gel or 30 min after lifting the cells to the air–liquid interface. One week after virus inoculation the cultures were harvested and typical cytopathic effects of HSV infection were seen only in cultures in which the epithelial cells were infected before lifting. DNA and RNA *in situ* hybridization were used to confirm the presence of HSV. In contrast, under these experimental conditions, no morphological changes were found in cultures infected after lifting on the air–liquid interface. Neither, infectious virus was recovered either from cells or culture supernatants. However, these cultures were positive for HSV DNA on PCR and showed expression of the latency associated transcript (LAT) gene by in situ hybridization and Northern blot (RNA) hybridization. Thus, both non-productive and lytic HSV infection could be produced *in vitro* and the outcome of the infection appeared to be dependent on the time of inoculation relative to epithelial differentiation.

In a follow up study, using the same model, it was demonstrated that application of HSV-1 before induction of differentiation at different time-points resulted in a productive infection, but the virus yield was found to be highest when infection took place at 72 h after seeding the cells (Hukkanen et al., 1999). Also, the effect of virus load [the amount of infectious virus varied from 0.1 to 5 PFU per culture (Vero cell infectivity)] on the outcome of HSV-1 infection was studied. Even at 0.1 PFU per culture, the HaCaT culture supernatants became HSV positive at 6 days post-infection and at 0.5 PFU or more per culture, a strong productive infection was detected. The effect of time of virus application on differentiation-induced cultures of HaCaT keratinocytes was investigated by infecting at 0.25–6 h after lifting the rafts to the air–liquid interface, i.e. after induction of stratification and differentiation. HSV-1 infection, detected by viral culture and immunohistochemistry, was observed in HaCaT raft cultures infected 15 min post-lifting. The cultures infected 30 min after lifting were negative for viral culture, and no HSV-1 antigen was detected by immunohistochemistry; however, HSV-1 DNA was detected by PCR and the presence of LAT was evidenced by in situ hybridization, indicating the presence of a non-productive infection. In cases where HSV-1 was applied onto the epithelium 60 min or more after lifting, two distinct patterns of virus spread were observed: lateral spreading on superficial layers of the epithelium, and a sharply demarcated infection throughout the thickness of the epithelium at the margins of the cultures (Hukkanen et al., 1999).

Visalli et al. (1997) have used organotypic foreskin and ectocervical epithelial cultures as a model to study the initial infectious process and spread of HSV-1 in fully stratified and differentiated human epithelial tissues. Infection of either foreskin or ectocervical tissues by wild-type HSV-1 (10^3 to 10^4 PFU per raft applied on top of the cultures) exhibited typical infection kinetics with eclipse, logarithmic, and plateau growth phases. Foreskin and ectocervical tissues were both able to support HSV-1 infection and exhibited similar replication kinetics, with virus titers of 10^7–10^8 PFU per infected culture obtained 48 and 96 h post-infection. Infection of fully stratified and differentiated epithelial tissues with a ribonucleotide reductase deletion mutant (ICP6Δ RR mutant) also exhibited eclipse, logarithmic, and plateau growth phases although 100-fold decrease in RR mutant virus titers compared to wild-type virus were obtained. Infection of the tissue was the result of virus penetrating the epithelial tissues from the top, since no virus was detected by titrations from tissues where the inoculum was applied underneath the tissue in the culture medium. Virus particles appeared to pass through gaps (microabrasions) in the suprabasal layers to infect the basal cells and initiate viral replication, similar to the *in vivo* situation. By 96 h post-infection with the wild-type virus, tissue demonstrating active HSV-1 infection had extensive morphological alterations lacking resemblance to stratified and differentiated epithelium. The virus was limited in its spread since not all the cells within the tissue had demonstrable infection. In contrast, the ICP6Δ HSV-1 mutant could infect and replicate in basal layers of the organotypic tissues but by 96 h post-infection minimal morphological alterations were demonstrated, indicating the inability of this mutant virus to spread to suprabasal layers of cells due to the differentiation process of the tissue.

Thus, studies of different types of HSV mutants in the organotypic raft cultures should allow defining the role of specific genes which are associated with infection and spread in a culture system that closely resembles the *in vivo* situation. Considering the lack of immune cells in this organotypic raft cultures, part of the limitation to viral spread appears to lie in the non-immune component (keratinocytes) of the epithelium. The high degree of polarization and formation of tight junctions seen in the organotypic epithelial tissues was suggested to contribute to the limitation of spread

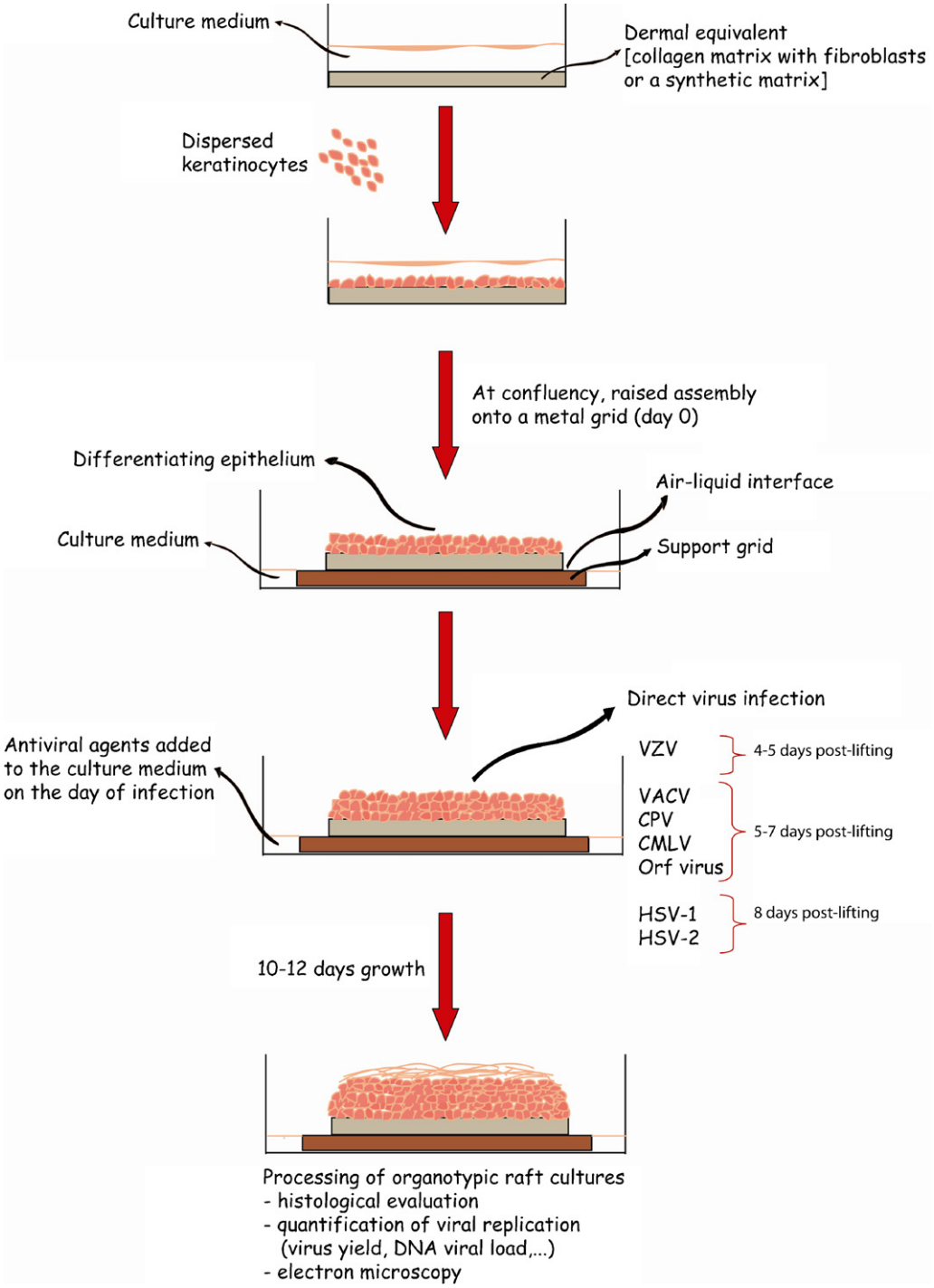

Fig. 3. Direct infection with α-herpesviruses or poxviruses of organotypic epithelial raft cultures for the evaluation of virus growth and activity of antiviral agents. Direct infection is performed by adding the viral inoculum directly on top of the rafts and the time of infection optimal for each virus was determined after performing kinetic experiments. Compounds are added to the culture media on the day of infection and this way of delivery can be considered as homologous to a systemic administration route. Following 10–12 days of growth, the raft cultures can be processed for histological evaluation, quantification of viral replication or electron microscopy.

by the virus. As shown previously by Hayashi (Hayashi, 1995), Madin-Darby canine kidney (MDCK) cells showed high infectivity and progeny production of HSV-1 before completion of polarization, while following polarization or formation of tight junctions, the infectivity and virus replication in MDCK cells was restricted significantly. Addition of Langerhan's cells or other immune cells to the organotypic raft cultures could be valuable to evaluate the role of the different types of cells of the skin that contribute to the limitations of viral spread.

We have employed raft cultures of PHKs to evaluate the activity of anti-herpesvirus compounds against HSV-1 and HSV-2 (Fig. 3) (Andrei et al., 2005). When the effect of the time of virus application on organotypic epithelial raft cultures was investigated, approximately 500 PFU of either HSV-1 or HSV-2 were applied on top of the rafts at various days after lifting the rafts at the air–liquid interface and fixed after 14 days post-lifting. Histological examination of the culture sections demonstrated typical HSV induced cytopathic effects, including ballooning and reticular degeneration

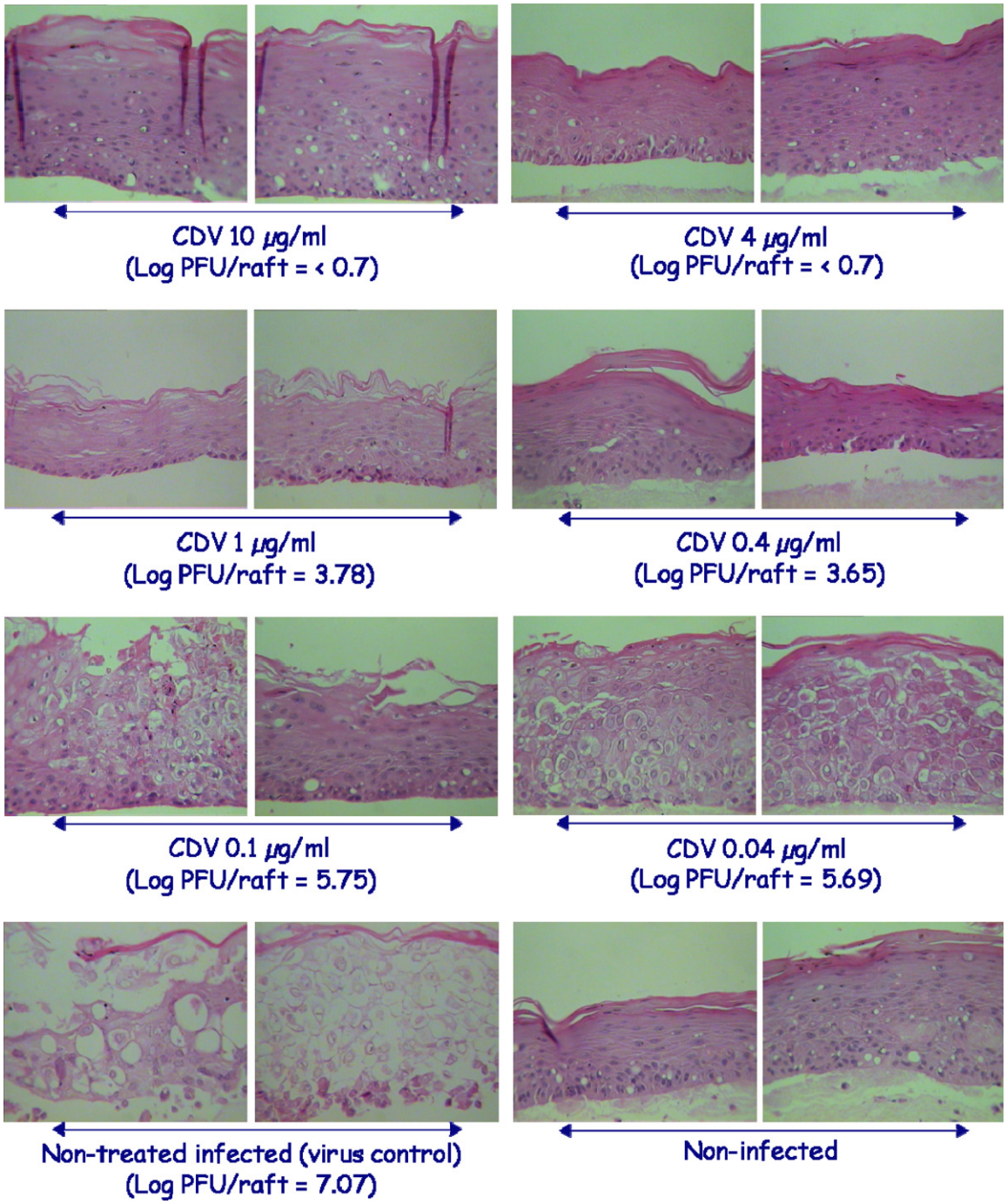

Fig. 4. Effects of cidofovir (CDV) on organotypic epithelial raft cultures of primary human keratinocytes infected with HSV-1 (Kos strain). Cultures were infected with approximately 500 PFU/raft at 8 days post-lifting. Different concentrations of the compound were added to the culture media on the day of infection and remained in contact with the cells till the rafts were fixed (at 12 days post-differentiation) for histological analysis or frozen for determination of virus production by plaque assay. Magnification: 40×.

of the keratinocytes together with the occurrence of intranuclear eosinophilic inclusion bodies, formation of characteristic intraepithelial vesicles, and multinucleation (Fig. 4). In cultures infected immediately after lifting or at 1, 2, 3, or 4 days post-lifting, all cells were infected and no epithelium could be seen due to the cytopathic effect of the virus. HSV infection was observed throughout the epithelium when the virus was added on the surface of a full-thickness epithelium grown for 6 or 8 days and the infection was not limited to a specific layer of the differentiated epithelium. Cultures infected 10 days after lifting exhibited cytopathic effects only in the upper layers of the differentiated epithelium.

The effects of specific anti-herpesvirus compounds against wild-type and thymidine kinase deficient (TK^-) HSV-1 and HSV-2 were evaluated following infection after 8 days of differentiation on top of the cultures and addition of the compounds in the culture media on the day of infection. Processing of the cultures for histology and viral quantification was performed 4 days after infection (i.e. 12 days post-differentiation). Acyclovir, penciclovir, and brivudin protected the epithelium against cyto-destructive effects induced by wild-type HSV-1 and HSV-2 viruses in a concentration dependent manner. Treatment with cidofovir or foscavir protected against the cytopathic effects induced by wild-type and TK^- HSV-1 and HSV-2

strains also in a dose-dependent mode. A correlation between the degree of protection as determined by histological examination and viral quantification as measured by infectious virus titers could be demonstrated for the different compounds (Fig. 4).

The discrepancies on spread of HSV in the differentiated epithelium observed among the different studies (Andrei et al., 2005; Hukkanen et al., 1999; Syrjanen et al., 1996; Visalli et al., 1997) may be due to (i) the type of cells used as feeders, (ii) the degree of proliferation of the epithelium produced, (iii) the virus inoculum used for the viral infection. The development of a normal, well-differentiated epithelium (where all the layers can be distinguished) can only be obtained if primary keratinocytes are employed. On the other hand, the type of feeder cells used is extremely important, since they may influence the quality of differentiation of the epithelium. It is recommended to use a slowly growing clone derived from mouse 3T3 cells as feeder cells to obtain a well-differentiated epithelium.

5.1.2. Investigations of varicella-zoster virus (VZV) growth in 3D epithelial raft culture

We have also demonstrated the feasibility of using organotypic epithelial raft cultures of human keratinocytes to study VZV growth and efficacy of antiviral agents (Fig. 3) (Andrei et al., 2005; McGuigan et al., 2007). Infection of the cultures with TK^+ and TK^- VZV strains led to morphological alterations, including ballooning of the cells. The VZV infection spread all along the epithelium, including the most superficial layers of the differentiated epithelium. However, the aspect of the lesions depended on the stage of differentiation of the raft cultures. The morphology of the epithelium was significantly altered when the virus was inoculated on top of the rafts up to 4 days after lifting, while the severity of the cytopathic effect was reduced when the virus was inoculated after 6 days, and even more after 8 days post-lifting. No cytopathic effect was noted when the infection was delayed to 10 days post-differentiation.

Different compounds have been evaluated against VZV in 3D cultures of human epithelial cells. Acyclovir, penciclovir, brivudin, CF1743 and its prodrug FV100 were able to inhibit the replication of the Oka strain (TK^+) but not of a TK^- strain, in a dose dependent manner, while cidofovir and foscavir proved equally active against both strains (Fig. 5). Due to the strong association of VZV with the cell membrane, the virus could not be released from cells by freezing and thawing of the epithelium. Therefore, the VZV-DNA was quantified by real-time PCR and for the different compounds, a close correlation between inhibition of viral replication and cytopathic effect was observed (Andrei et al., 2005; McGuigan et al., 2007).

T cells and skin can be considered as critical targets for VZV pathogenesis since T cells appear to be a major target cell for VZV viremia, and these migrating cells are responsible for transport of the virus from the respiratory epithelial inoculation sites to dermal and epidermal cells. Also, VZV skin lesions contain high titers of infectious virus, which is transmissible to other susceptible individuals in the population. However, the cells generally employed for culturing and studying VZV growth are human fibroblasts. Human epithelial cells are a more relevant system than fibroblasts to investigate VZV pathogenesis and for evaluating antiviral agents. In addition, considering the lack of a simple small-animal model to study VZV, the organotypioc epithelial raft cultures of human epithelial cells emerges as the closest system to the natural situation available to study new investigational drugs.

5.2. β-Herpesviruses

Human cytomegalovirus (HCMV), the prototype member of the beta-herpesvirusvirinae, infects between 30% and 70% of the population in developed countries and >90% in the developing world. Like other herpesviruses, following primary infection in the normal host, the virus remains in a latent state. Primary infection in immunocompetent individuals is usually benign, with minimal or no clinical manifestations, except for some cases of mononucleosis-like syndrome. Immunocompetent persons do occasionally experience severe symptoms on viral reactivation, although quite severe disease even in immunocompetent hosts may not be as rare as previously thought (Rafailidis et al., 2008). A reduction in immune surveillance, as for example in the context of immunosuppression in solid organ transplant (SOT) or hematopoietic stem cell transplant (HSCT) recipients, in cancer patients undergoing chemotherapy and in individuals with AIDS favors reactivation of the virus. HCMV infections in the immunocompromised host (either primary infection, reactivation from latency, or re-infection) are a leading cause of morbidity and mortality (Fishman et al., 2007; Hodson et al., 2005). Furthermore, HCMV infection of the human embryo, a host with immature immunological responses, is often associated with serious complications (Nassetta et al., 2009). Congenital CMV infection is estimated to affect 1–3% of infants annually and remains an important public health problem causing significant morbidity and mortality.

HCMV infects many different cell types within the host, including endothelial cells, monocytes-derived macrophages, smooth muscle cells, polarized epithelial cells, fibroblasts, T-cells, granulocytes, stromal cells, neuronal cells and hepatocytes (Jarvis and Nelson, 2002; Sinclair, 2008). Recent studies have implicated endothelial cells and specific cell types of the myeloid lineage [CD34+ hematopoietic progenitors, CD33+ granulocyte-macrophage progenitors and monocyte-derived macrophages] as critical sites of HCMV persistence and latency (Reeves and Sinclair, 2008).

Although HCMV disease in an immunosuppressive setting usually presents as visceral disease ranging from pneumonia to various other, widely disseminated diseases, cutaneous manifestations are rare compared with the prevalence of HCMV infection of other organs. Most of the skin lesions in which HCMV was found were ulcers localized mainly on the anogenital areas (AbdullGaffar et al., 2008; Lee, 1989; Lesher, 1988). Drago et al. (2000) divided HCMV skin lesions into non-specific generalized lesions, e.g. maculopapular eruptions, especially after antimicrobial treatment, and more specific lesions, which can be either localized, e.g. ulcerations, or generalized, e.g. vesiculobullous eruptions. Specific cutaneous lesions appear to arise from infection of the endothelium of cutaneous blood vessels during the viremic phase of the illness and the cells infected by HCMV are mostly mesenchymal cells (e.g. vascular endothelial cells, fibroblasts, and macrophages) and only rarely epithelial cells (e.g. epidermal keratinocytes and sweat gland epithelial cells) (AbdullGaffar et al., 2008).

HHV-6 and HHV-7 belong to the Roseolovirus genus of the β-herpesviruses, and the HHV-6 species are divided in two variants: HHV-6A and HHV-6B (Zerr, 2006). Both viruses are ubiquitous and infection occurs during infancy. Infections caused by both viruses can be asymptomatic or can manifest as mild, febrile illness such as roseola (also known as exanthema subitum). HHV-6 is the principal etiological agent of roseola; HHV-7 can also be responsible for 10–31% of roseola-like illnesses and has been reported to have a strong association with pytiriasis rosea (Caselli and Di Luca, 2007). HHV-6 and HHV-7 are also recognized as important pathogens in immunocompromised hosts when reactivation of the virus occurs (Yoshikawa, 2003).

During primary infection, HHV-6 and HHV-7 are found in high concentrations in peripheral blood mononuclear cells. HHV-6 can also be found in lymph nodes, kidney (tubular epithelial cells,

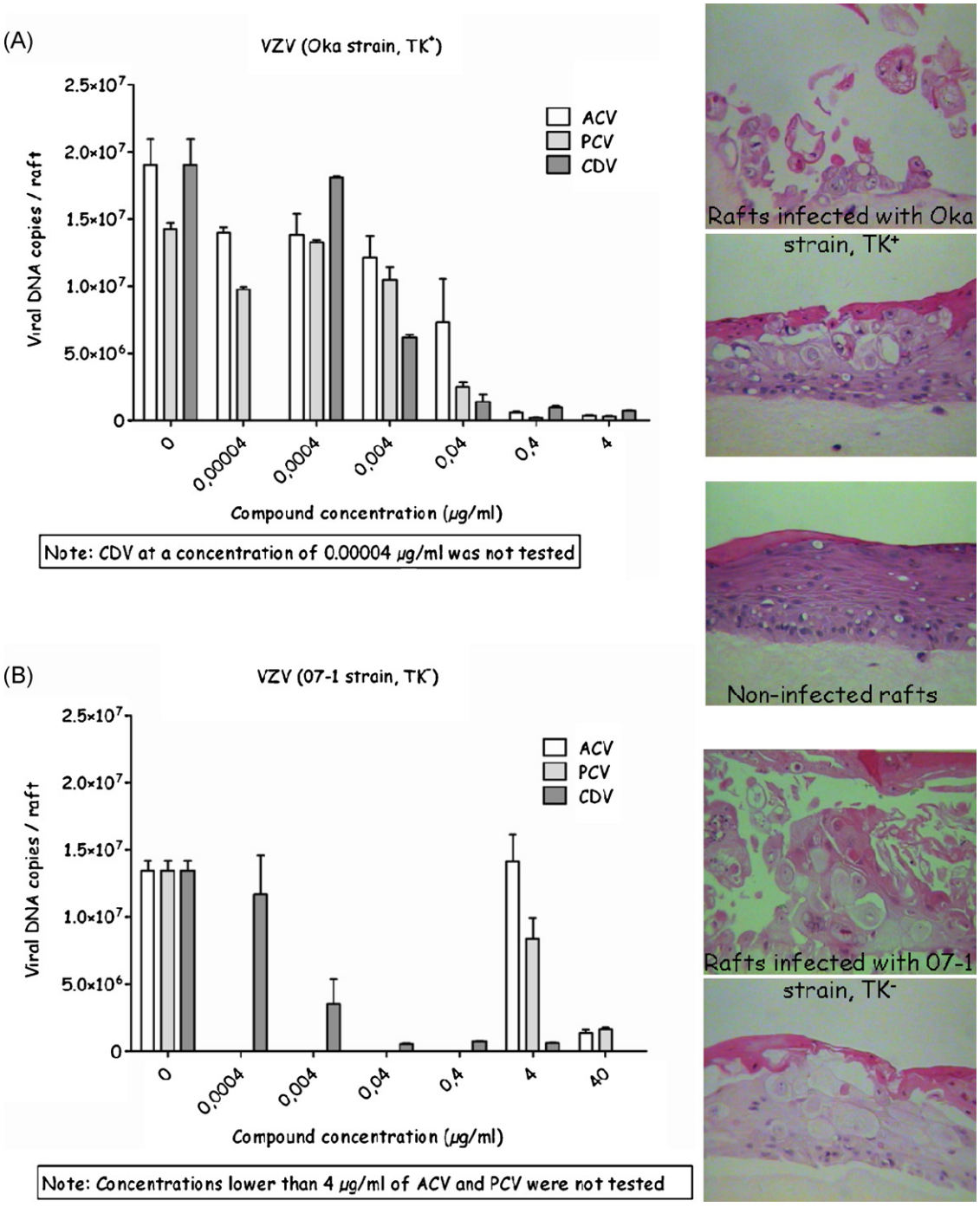

Fig. 5. Effects of acyclovir (ACV), penciclovir (PCV), and cidofovir (CDV) on organotypic epithelial raft cultures of primary human keratinocytes infected with varicella-zoster virus (VZV). The cultures were infected at 5 days post-lifting with approximately 500 PFU of the Oka strain (thymidine kinase wild-type, TK^+) or the 07-1 strain (TK-deficient, TK^-). Compounds were added to the culture media on the day of infection and remained in contact with the cells till the rafts were fixed and processed for histological analysis (A) or frozen for DNA isolation and quantification of VZV-DNA by real-time PCR (B). Magnification: 40×.

endothelial cells and histiocytes), salivary glands and central nervous system tissues, where the virus has been localized to neurons and olygodendrocytes (Komaroff et al., 2006; Kondo et al., 2003; Mori, 2009). So far, two cell types have been recognized as sites of HHV-7 infection *in vivo*, including CD4+ T-lymphocytes and epithelial cells in salivary glands. Saliva is considered the major source of transmission for both viruses. The sites of HHV-6 and HHV-7 latency are not completely defined. HHV-6B appears to establish latency predominantly in the peripheral blood monocytes/macrophages and in hematopoietic progenitor cells. A cell population in the peripheral blood that might harbor latent HHV-6A or HHV-7 has not been identified yet.

So far, no studies in a differentiated epithelium have been performed with β-herpesviruses. Considering that these viruses can be transmitted through the saliva, and are able to infect epithelial cells in some organs, it would be worth using organotypic epithelial raft cultures of primary human keratinocytes derived from mucosal epithelia to investigate the cellular microenvironment, the virus determinants of epithelial cell tropism, and the mechanisms of viral transmission.

5.3. γ-Herpesviruses

Epstein-Barr virus (EBV) is an orally transmitted virus that has a worldwide distribution, infecting more than 90% of the human population (Cohen, 2000). Primary infection occurs in the oropharyngeal epithelium, which is permissive for virus replication. B lymphocytes are then infected by progeny virions and the virus establishes a lifelong latency in the B-cell compartment. Periodic reactivation may occur with reinfection of the oropharyngeal epithelium, virus release, and transmission of virus via the salivary route. Primary infection is usually asymptomatic and only when it is delayed until adolescence or adulthood a benign lymphoproliferative disease, known as infectious mononucleosis, may occur. Long-term carriage of the virus is also implicated in the development of immunoblastic lymphoma, Burkitt lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, oral hairy leukoplakia and gastric carcinoma (Pattle and Farrell, 2006; Takada, 2001; Thompson and Kurzrock, 2004; Thorley-Lawson and Gross, 2004). These are diseases that reflect the predominant tropism of EBV for two distinct cell types: B lymphocytes and stratified epithelium (Fafi-Kremer et al., 2005; Frangou et al., 2005; Herrmann et al., 2002; Walling et al., 2001). Infection of B lymphocytes is usually non-productive or latent, whereas intermittent reactivation and virus replication in epithelial surfaces allows the spreading of the virus to new hosts (Amon and Farrell, 2005; Borza and Hutt-Fletcher, 2002; Dolcetti, 2007). In EBV latency, only a subset of genes is expressed and different types of latency can be distinguished according to the latent gene products expressed.

Kaposi sarcoma associated herpesvirus (KSHV) or human herpesvirus 8 (HHV8) is etiologically linked with Kaposi sarcoma (KS), a multifocal endothelial cell tumor most commonly seen in AIDS patients (Dictor et al., 1996). KS lesions are characterized by the presence of spindle-shaped endothelial cells and inflammatory cells (Aluigi et al., 1996; Dictor et al., 1996). In KS lesions latent virus predominates, with a low percentage of cells exhibiting lytic replication. Several lines of evidence point to a central role of KSHV not only in the pathogenesis of KS but also in the pathogenesis of two B cell-proliferative disorders, primary effusion lymphoma (PEL) and multicentric Castelman's disease. Epidemiological data suggest that both sexual and non-sexual routes of transmission of KSHV are possible. As seen with other herpesviruses such as cytomegalovirus, HHV-6, HHV-7 and EBV, the oral cavity has been identified as the major site for the shedding of KSHV (Boldogh et al., 1996; Casper et al., 2004, 2007; Koelle et al., 1997). Activation of KSHV lytic gene expression was recently shown to occur as keratinocytes differentiate into a mature epithelium, and this may be responsible for the presence of infectious KSHV in saliva (Johnson et al., 2005). Cell types identified as supporting KSHV lytic and/or latent gene expression include monocytes, endothelial/spindle cells of KS lesions, B cells, and epithelial cells (Blasig et al., 1997; Cannon et al., 2000; Diamond et al., 1998; Dupin et al., 1999; Lagunoff et al., 2002; Sturzl et al., 1997). Both latent and lytic gene expression are considered to have a role in diseases associated with KSHV but the factors affecting lytic or latent gene expression are not well defined (Lu et al., 2005; Qin et al., 2008; Varthakavi et al., 2002; Vieira et al., 2001; Zeng et al., 2007).

5.3.1. Investigations of Epstein-Barr virus (EBV) in 3D epithelial raft culture

Due to the ability of EBV to infect epithelial cells, organotypic epithelial raft cultures have been used to study the effects of individual EBV genes when expressed in an epithelial environment.

The BHRF1 gene encodes a 17-kDa protein with both sequence and functional homology to the antiapoptotic Bcl-2 oncogene. BHRF1 behaves like Bcl-2 in preventing cells from apoptosis induced by a range of stimuli. The effect of BHRF1 on epithelial cell growth and differentiation in comparison to Bcl-2 was analyzed by using stable transfection of SCC12F cells, an immortalized but non-tumorigenic epithelial cell line derived from a squamous cell carcinoma of facial epidermis (Dawson et al., 1998). These cells retain several characteristics unique to normal epidermal keratinocytes, including responsiveness to terminal differentiation signals. Both BHRF1 and Bcl-2 inhibited epithelial differentiation in the raft cultures; however, the effect of Bcl-2 was more pronounced, resulting in an almost complete blockade of differentiation. On the other hand, BHRF1-expressing SCC12F cells proliferated at a much higher rate than cells expressing Bcl-2. This effect was supported by the fact that BHRF1, but not Bcl-2, promoted rapid transit through the cell cycle. These data point to important differences between BHRF1 and Bcl-2 and suggest that BHRF1 may function to promote the survival and proliferation of lytically infected cells. This proliferative property of BHRF1 suggests that this protein may serve to increase the susceptibility of virus-infected cells to oncogenic transformation, and thus contributing to the development of virus-associated tumors.

EBV has three latent membrane proteins (LMPs), i.e. LMP1, LMP2A, and LMP2b, which are expressed in nasopharyngeal carcinoma. Organotypic epithelial raft cultures have been used to investigate the effects of expression of EBV LMPs in keratinocyte differentiation and cell adhesion (Dawson et al., 1990). SCC12F cells were stable transfected with the EBV gene encoding LMP. LMP expression was accompanied by changes in the epithelial cell surface phenotype that resembles surface changes observed in nasopharyngeal carcinoma. LMP-expressing cells produced a much thicker, but less organized epithelium with poorer intercellular contacts when grown in 3D raft cultures. Specific differences between control transfectants and LMP-transfectants could be observed following staining for desmosomes, a marker of interepithelial cell contact and staining for involucrin, a marker of epithelial differentiation.

In another study, an HPV-immortalized cell line FEP 1811, that demonstrate normal stratification with keratinization was used to transfect with a retrovirus vector containing the EBV LMP1, LMP2a, or LMP2b genes (Farwell et al., 1999). Cells expressing LMP2a or LMP2b were defective in their ability to mature and progress through normal squamous stratification when compared to the parental cell lines. Cells expressing LMP1/LMP2a demonstrated "pseudoinvasion" into the raft dermal equivalent. In addition, in LMPs-expressing cell lines there was a consistent and dramatic up-regulation in the suprabasal layers of expression of laminin 5 and α6β4 and β1 integrins. ICAM-1 (intercellular adhesion molecule type 1) was not expressed in the control cell lines but it was up-regulated in the LMPs-positive cells grown in the raft culture system. Also, expression of α3 and α5 integrins was also up-regulated in the LMP-expressing cell lines, while a loss of the normal basal layer expression was noted with α2 integrin. No changes in the patterns of expression of E-cadherin and desmoplakin were observed. This study indicated that LMP2A and LMP2b alone, and LMP1/LMP2a co-expressed, are capable of altering keratinocytes cell adhesion expression consistent with nasopharyngeal carcinoma.

In a later study, the LMP2a protein was expressed in the human keratinocyte cell line HaCaT, which exhibits many features of normal stratified epithelium when cultured in organotypic rafts (Scholle et al., 2000). LMP2a was expressed throughout the time in the culture and in all cell layers. Raft cultures derived from LMP2a-expressing HaCaT cells showed morphology strikingly different from that of vector control rafts. The LMP2a-expressing rafts were thickened and the cells were more rounded with large nuclei in comparison to flattened appearance of vector control cells. The overall thickening of the LMP2a-expressing raft cultures reflected an increased rate of proliferation as demonstrated by BrdU

incorporation and detection of DNA-synthesizing cells by immunohistochemistry. Single scattered BrdU-positive cells were detected in the vector control cultures, whereas the number of proliferating cells was greatly increased in the LMP2a-expressing rafts. In addition, the proliferating cells were not restricted to the basal cell layer, as expected for normal differentiated epithelia, but were present in all cell layers. Also, involucrin expression was not observed in the LMP2a-expressing rafts. Involucrin, a suprabasal precursor protein of the cornified envelope, is expressed in stratified epithelia and is indicative of squamous cell proliferation. Thus, the lack of involucrin expression together with the absence of enucleated cells in the top layers of LMP2a-expressing rafts and the presence of proliferating suprabasal cells indicated that LMP2a expression interferes with the ability of epithelial rafts to differentiate. Expression of LMP2a and LMP2b was also associated with an increased capacity to spread and migrate on extracellular matrix (Allen et al., 2005).

5.3.2. Investigations of HHV-8 (KSHV) virus in 3D epithelial raft culture

Since epithelial cells support KSHV replication *in vivo*, Johnson et al. (2005) developed an organotypic raft culture model that employed keratinocytes isolated from normal tonsils in order to test if KSHV uses the differentiation of oral epithelium as a mechanism for activation of lytic gene expression and virus production. This hypothesis was based on the fact that the oral cavity has been identified as the major site for the shedding of infectious KSHV and viral DNA is frequently detected in the saliva of KSHV seropositive patients although the virus does not appear to replicate in the salivary glands. Raft cultures of keratinocytes derived from human tonsils produced a non-keratinized stratified squamous oral epithelium *in vitro* as demonstrated by the presence of nucleated cells at the apical surface, the expression of involucrin and keratins 6, 13, 14, and 19, and the absence of keratin 1. A recombinant virus that expresses the green fluorescent protein during latency from the cellular EF-1α promoter and the red fluorescent protein (RFP) during lytic replication from the viral early PAN promoter was used to examine KSHV lytic gene expression during epithelial differentiation. Infection of keratinocytes with this recombinant virus resulted in latent infection; however, when the keratinocytes were allowed to differentiate into a multilayered epithelium, lytic cycle activation occurred, as evidenced by RFP expression, the expression of late virion proteins, and the production of infectious virus at the epithelial surface. These findings indicated that KSHV lytic activation occurs as keratinocytes differentiate into a mature epithelium, and it may be responsible for the presence of infectious virus in the saliva.

6. Adenoviruses

Replication of competent adenoviruses are being investigated as oncolytic agents in clinic, especially with neoplasias of epithelial origin, such as head and neck and prostate cancers (DeWeese et al., 2001; Nemunaitis et al., 2000). Exclusive virus replication in cancer cells has been proposed as a safer way to develop oncolytic adenoviruses. Two major approaches have been developed to achieve tumor-specific replication. One of these approaches is based on the control of adenovirus early gene expression by a tumor-specific antigen promoter (Alemany et al., 1999; Rodriguez et al., 1997; Yu et al., 1999). In another method to restrict adenovirus replication to tumor cells, viral genomic deletions that affect viral protein functions dispensable in cancer cells are introduced (Bischoff et al., 1996; Fueyo et al., 2000). The demonstration of replication selectivity has proven difficult due to the lack of an *in vitro* system to study adenovirus replication in a physiological setting. The systems to study oncolytic adenovirus are mostly based on the use of primary or transformed cell lines grown as monolayer cultures or as xenografts in nude mice. However, differences in the physical characteristics of epithelial tissue and monolayer cell cultures could have important consequences for the infectivity of the virus. In this regard, the protective cornified layer of a stratified epithelium may act as an effective barrier against adenovirus infection and the distribution of the coksackie-adenovirus receptor (CAR) appears to be heterogeneous across the epithelium (Huber et al., 2000; Hutchin et al., 2000). The efficiency of adenovirus-mediated gene transfer to oropharyngeal cells was shown to be correlated with cellular differentiation and human CAR expression (Hutchin et al., 2000). Studying the viral life cycle in a stratified human epithelium looks like the situation during clinical gene transfer. The organotypic epithelial raft cultures allowed the analysis of the life cycle of adenovirus in stratified epithelia that more closely resemble the *in vivo* situation.

6.1. Epithelial raft cultures for the study of adenoviruses

Several adenovirus mutants have been investigated for their potential to conditionally replicate (conditionally replicating adenoviruses, CRAds) and promote the killing of cells expressing human papillomavirus (HPV) E6 and E7 oncoproteins, which are present in a high percentage of anogenital cancers (Balague et al., 2001). In this study, an organotypic model of human stratified squamous epithelium derived from primary keratinocytes engineered to express HPV-18 oncoproteins stably was used. Wild-type adenovirus was shown to promote a wide spread cytopathic effect in all infected cells, while E1A- and E1A/E1B-deleted adenoviruses caused no deleterious effect regardless of the coexpression of HPV18 E6E7. An adenovirus deleted in the CR2 domain of E1A, necessary for the binding to the pRB family of pocket proteins, showed no selectivity of replication since it equally killed normal keratinocytes and E6E7-expressing keratinocytes. However, an adenovirus mutant deleted in the CR1 and CR2 domains of E1A exhibited preferential replication and cell killing of HPV E6E7-expressing cultures. Thus, the organotypic culture represents a distinct model to evaluate adenovirus selectivity for tumor cells.

In a later study, the same group of investigators further analyzed the infectious cycle of adenovirus in organotypic cultures of primary human keratinocytes (Noya et al., 2003). Wounding of the epithelium was required for the infection to occur. In addition, adenovirus infection appeared to initiate at the basal or parabasal cells that express the high-affinity CAR receptor, while the productive phase takes place in differentiated cells. This appears to be due, at least in part, to the differentiation-dependent activation of the E1A and E2A early promoters and E4 promoters. Similarly to HPV, adenovirus infection triggered a response mediated by the abnormal accumulation of cyclin E and p21cip1 proteins.

In a more recent study, Heideman et al. (2005) described a new CRAds with efficient and selective oncolytic replication in HPV-containing E6 and E7 proteins. To enhance its oncolytic potency, they modified the AdCB016 virus (which exhibits preferential replication in HPV E6/E7-expressing keratinocytes) to express a p53 variant resistant to degradation by HPV E6 protein. This new oncolytic virus exhibited 10–1000-fold greater efficacy than the AdCB016 on high-risk HPV-positive cervical carcinoma cells and HPV-immortalized keratinocytes in monolayer cultures and in organotypic epithelial raft cultures.

7. Parvoviruses

The members of the family *Parvoviridae* are small (diameters of 18–26 nm), non-enveloped viruses. They have isometric nucleocapsids that contain a molecule of linear, negative-sense or positive-sense, single-stranded DNA (genome size: 4000–6000 nucleotides). This family includes viruses that are pathogenic for

humans, i.e. human parvovirus B19 (subfamily *Parvovirinae*, genus *Erythrovirus*), the causative agent of fifth disease, hydrops fetalis, and aplastic anemia, in particular in patients with preexisting hematopoietic disease and human bocavirus (sub-family *Parvovirinae*, genus *Bocavirus*), the fourth most frequently detected virus among symptomatic children with respiratory infection. Other human parvoviruses of interest include the five current species of human adeno-associated viruses (AAV), which resides in the genus *Dependovirus*. As the name implies, the AAVs rely on another "helper" virus to replicate. Members of the adenovirus and herpesviruses families have been well documented to serve as helper viruses for AAVs. In the absence of helper virus, AAV maintain itself latently in the host cell by chromosomal integration at a favored site. Eighty to 90% of the human adult population is seropositive for AAV infection and thus far there has been no disease state or pathology associated with AAV infection.

7.1. Epithelial raft cultures for the study of parvoviruses

Meyers et al. (2000) described for the first time the utility of using the differentiated epithelial tissue system to provide a proper environment for the complete life cycle of the adeno-associated virus type 2. Interestingly, when primary foreskin keratinocyte monolayers grown on collagen matrices were infected with AAV while still submerged in medium and then raised to the air–liquid interface (to allow grow and differentiation into epithelial tissue), AAV underwent its complete life cycle without a helper virus. AAV progeny production correlated with epithelial differentiation, since AAV was unable to replicate without a helper virus in non-differentiating keratinocytes. Viral particles could be detected in the granular layers of the organotypic epithelial rafts by electron microscopy. Furthermore, histological changes indicative of cytopathology induced by AAV were observed. Thus, the authors have provided a new biological model for AAV, i.e. AAV as an epithelial-tropic autonomous parvovirus that can modify normal squamous differentiation.

Since the natural host tissue for AAV is skin, in which it functions as an autonomous parvovirus, AAV type 2 (AAV-2) can be considered as a useful vector for skin gene therapy. Agrawal et al. (2004) demonstrated the utility and efficiency of AAV-based gene delivery to produce genetically altered keratinocytes and recombinant skin. In this study, the researchers demonstrated that recombinant AAV-2 vectors carrying the granulocyte-macrophage colony-stimulating factor (GM-CSF), HPV E6, or green fluorescent protein (GFP) transgene could transduce primary human keratinocytes in organotypic epithelial rafts.

8. Poxviruses

Poxviruses are large, enveloped DNA viruses and unlike most other DNA viruses, poxviruses replicate entirely in the cytoplasm. They cause a variety of diseases of veterinary and medical importance. Poxviruses have been shown to enter their hosts either by the respiratory route or through the skin. Natural infection with many poxviruses leads to the characteristic skin "pox" lesions, and the severity of the infection varies dramatically from one species to another, ranging from a local, self-limiting infection to a devastating systemic disease, such as smallpox.

Humans can be infected by viruses belonging to *Orthopoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, and *Yatapoxvirus* genera, but it is the Orthopoxviruses variola and monkeypox that are of primary concern. Variola virus (VARV) causes smallpox, which was eradicated in the 1970s using ring containment methods and immunization with vaccinia virus (VACV) (Fenner, 1982, 1993). Human cases of monkeypox still occur in parts of central Africa, where monkeypox virus infects a rodent reservoir, and have been exported to North America by the exotic pet trade (Di Giulio and Eckburg, 2004; Kile et al., 2005). Although uncommon, the rare zoonotic infections with cowpox virus (CPV) can pose a serious health risk to human beings, particularly among young children and immunocompromised persons (Vorou et al., 2008). Orf virus is the prototype of the *Parapoxvirus* genus; it is the causative agent of a worldwide contagious skin infection of sheep and goats known as contagious ecthyma, contagious pustular dermatitis, or scabby mouth. Although sheep and goats are the natural hosts for orf virus, the infection can also arise in humans. Orf virus infection occurs frequently in rural areas and among individuals within occupational categories at risk (farmers, sheep shearers, and veterinarians). Orf virus has a high epitheliotropism, and it develops a local epidermal infection by replicating in the germinal layer of the epithelium. The only other poxvirus disease, commonly seen in humans, is that caused by molluscum contagiosum virus (MCV). MCV infections are rarely serious in healthy persons, although the disease can be troublesome when immunity is compromised (Brown et al., 2006; Tyring, 2003).

8.1. Investigations of poxvirus gowth in 3D epithelial raft cultures

As keratinocytes are one of the main targets for productive infection of poxviruses, we have adapted the organotypic epithelial raft cultures to study the replication of different orthopoxviruses, including VACV, CPV, and camelpox virus (CMLV) (Duraffour et al., 2007a,b; Lebeau et al., 2006; Snoeck et al., 2002), which has been shown to be the closest known orthopoxvirus to VARV (Fig. 3). The cytopathic effects obtained following infection with the different orthopoxviruses proved to be characteristic of those seen in clinical lesions induced by orthopoxviruses, i.e. the presence of inclusion bodies. The addition of the selected ANPs to the culture media of infected rafts led to inhibition of virus growth as monitored by histological examination of virus-induced cytopathic effects and/or production of infectious virus (Fig. 6). It should be noted that the 3D epithelial raft culture model appeared to be a more relevant and appropriate model to study the effect of antiviral agents than monolayers of primary keratinocytes. Indeed, the selectivity of antiviral agents [measured as the ratio CC_{50}/EC_{50}, where CC_{50} is the 50% cytotoxic concentration for growth cell and EC_{50} is the 50% effective antiviral concentration] in 2D cultures of primary human keratinocytes resulted to be low compared to 3D differentiated epithelial cells. This may be explained by the fact that keratinocytes normally grow and differentiate, and culturing them in monolayers and treating them with antiviral drugs may increase the stress and toxicity of the compounds.

Also, we demonstrated that the 3D epithelial raft cultures could be applied to study animal poxviruses in their natural host epithelial cells. In this regard, primary ovine keratinocytes were employed to study the growth of the parapoxvirus orf and the effect of antiviral agents (Dal Pozzo et al., 2005). Orf virus could replicate in the ovine raft cultures, giving a histological picture similar to that in infected skin *in vivo* (Fig. 7); in this system a correlation between the histological features and viral replication was demonstrated. Furthermore, this system has proved to be a reliable model for the study of the antiviral activities of several compounds.

In the different studies considering antiviral activities of selected compounds against pox- and herpesviruses, the compounds were delivered to the rafts by addition to the culture medium; therefore, the drugs are entering the rafts from what could be considered the equivalent of the basal membrane. Thus, the basal delivery of the compounds might mimic a systemic delivery route with diffusion into the dermis. Further experiments are necessary to adapt the organotypic epithelial raft culture system as a model to mimic topical delivery of compounds via application of drugs to the top of the epithelium.

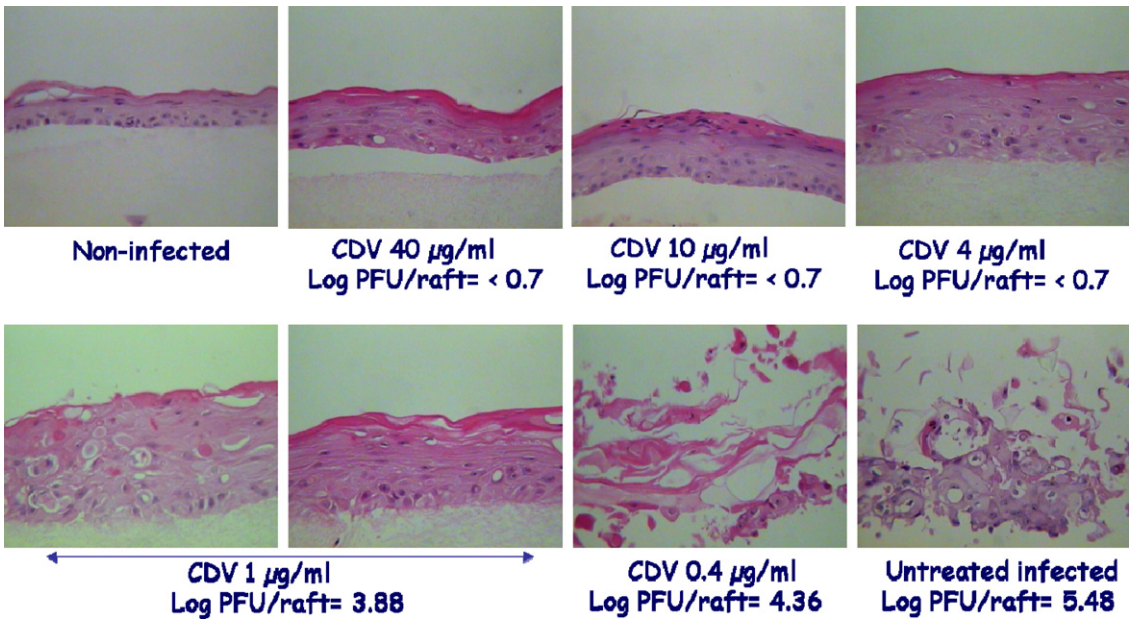

Fig. 6. Effects of cidofovir (CDV) on organotypic epithelial raft cultures of primary human keratinocytes infected with VACV (Lederle strain). Cultures were infected with approximately 500 PFU/raft at 7 days post-lifting. Different concentrations of the compound were added to the culture media on the day of infection and remained in contact with the cells till the rafts were fixed for histological analysis or frozen for determination of virus production by plaque assay. Magnification: 40×.

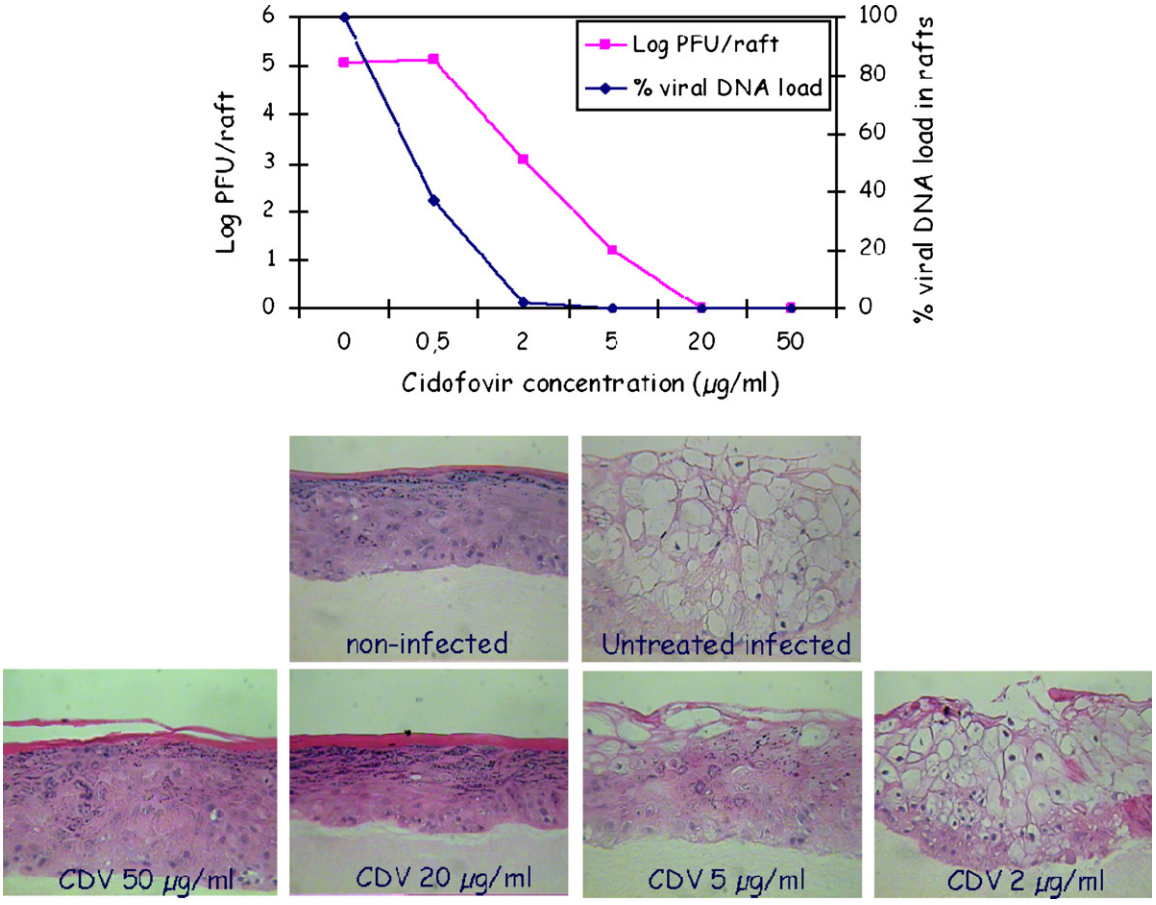

Fig. 7. Effects of cidofovir (CDV) on organotypic epithelial raft cultures of primary lamb keratinocytes infected with orf virus (NZ2 strain). Cultures were infected with approximately 500 PFU/raft at 5 days post-lifting and different concentrations of the compounds were added to the culture media on the day of infection. After 12 days post-lifting of the rafts, the cultures were either fixed for histological analysis or frozen for determination of virus production by plaque assay and viral DNA quantification by real-time PCR. Magnification 40× (Dal Pozzo et al., 2005).

9. Conclusions

Organotypic epithelial raft cultures represented a breakthrough in the study of papillomaviruses due to the strict link of HPV replication with epithelial cell differentiation. However, many other viruses target epithelial cells, representing the initial site of infection, the site of replication, and a staging area for transportation to other tissues. Therefore, it is not surprising that in the last years the use of organotypic epithelial raft cultures has been expanded to other viruses (i.e. herpesviruses, adenoviruses, poxviruses, and parvoviruses) able to infect epithelial cells at least during a part of their replicative cycle. Not only the replication of HPV but also that of HHV-8 and AAV-2 has been shown to be dependent on epithelial cell differentiation, making the organotypic epithelial raft cultures an essential tool for the study of these viruses. We have demonstrated that organotypic epithelial raft cultures can be adapted to study the efficacy and selectivity of antiviral agents against α-herpesviruses and several orhtopoxviruses. To date, we have evaluated the efficacy of different antivirals by examining the virus-induced cytodestructive effects following hemathoxylin–eosin stains of raft sections. The quantification of the antiviral effects has been performed by measuring viral titers by plaque assay and/or by measuring viral DNA load by real-time PCR. Other imaging methods such as two-photon microscopy (a fluorescence imaging technique that allows imaging living tissue) could be used to explore virus growth and dissemination within these cultures.

The study of some viruses such as molluscum contagiosum virus (MCV) has been hampered by the lack of an *in vitro* system to replicate the virus. MCV, the only member of the *Molluscipox* genus, is present worldwide and is directly passed by direct skin contact to produce cutaneous and, rarely, mucosal lesions. Considering the strict tropism of MCV for epithelial cells, it seems imperative to investigate the feasibility of studying MCV growth in 3D cultures of PHKs. A typical cytopathic effect (characterized by the appearance of huge infected cells, with internal organelles dislocated and obliterated by a large intracytoplasmic inclusion, i.e. Henderson-Patterson inclusion bodies or Molluscum bodies) was observed when fresh skin lesions recovered from preadolescent children were cut in very tiny pieces and were placed on top of the raft cultures which were incubated for a period of 25 days (our unpublished data).

OERCs can also be adapted to reproduce the process of viral transmission from blood to epithelial cells by studying the ability of mononuclear cells (MCs) to transmit the viral infection to a differentiated epithelium. Indeed, we have recently assessed the feasibility of using HSV- or VACV-infected MCs to transfer the viral infection to organotypic epithelial raft cultures (Andrei et al., 2009a). Furthermore, specific populations of MCs and endothelial cells can be added to the 3D culture system. An immune reconstituted epithelium represents a relevant *in vitro* model for the study of viral immunobiology.

Although the preparation of 3D cultures of epithelial cells is time consuming and laborious, they provide an *in vitro* system to study virus growths and virus–host cell interactions under conditions that more closely resemble the *in vivo* situation. In addition, organotypic epithelial raft cultures are essential for the study of virus whose grows is dependent on epithelial cell differentiation.

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