Susceptibility of human retinal pigment epithelial cells to different viruses

Abstract

Background: Different viruses have been reported to be involved in retinal diseases in animal systems. In humans, herpes simplex virus and cytomegalovirus have been found to cause retinal disease. Most of the studied viruses are neurotropic. In this study, the in vitro susceptibility of human retinal pigment epithelial cells (RPEC) to representative members of different groups of human pathogenic viruses was investigated.

Methods: Early cultures of RPEC - after two or three passages - were infected with the following viruses: herpes simplex virus (HSV) type 1, human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV), cytomegalovirus (CMV), adenovirus types 1 and 7, measles virus, parainfluenza virus and coxsackie virus B3.

Results: Cultures of RPEC could be infected with neurotropic viruses like HSV or measles virus as well as with typical respiratory viruses like parainfluenza or adenoviruses. Coxsackievirus, an enterovirus, replicated as well as human CMV, whereas EBV and HHV-6, two lymphotropic viruses, failed to infect RPEC.

Conclusion: These findings suggest that a variety of viruses, including those causing rather common illnesses, might be capable of inducing retinal lesions under certain circumstances due to haematogenous spread during the course of viraemia.

Introduction

A variety of pathogens have been described to affect the retinal tissue of different species of laboratory animals. Most studies have investigated known neurotropic viruses.

Herpes simplex virus (HSV) is among the best studied [25, 41]. Among the animal viruses, rare neuropathogens like bornavirus have been reported – a horse or sheep RNA virus which induces multifocal retinorchoriopathy in experimentally infected rabbits [21]. Measles virus and SSPE (subacute sclerosing panencephalitis) strains of measles virus have been inoculated intracerebrally into suckling hamsters [26] or rats [28]. Lymphocytic choriomeningitis virus (LCM) causes disease in newborn rats [5]. Rabies virus has been studied in the visual system of rats [6], Semiliki Forest virus in mice [27], and bluetongue virus in chicken embryos [39].

Infections with murine cytomegalovirus (CMV) lead to severe chorioretinal lesions in mice [1], as does bovine diarrhoea virus in cattle [3]. Vesicular stomatitis virus has also been tested in the mouse system [24]. A murine coronavirus, the mouse hepatitis virus, induces retinal degenerative disease in BALB/c mice [30] and has been shown to establish persistent infection in murine retinal cell culture [40]. Rift Valley fever virus [37] and Sindbis virus [4] have also been found to be involved in ocular immunopathology.

A molecular clone of HIV has been transfected into human fetal retinal cultures containing both glial and neuronal cells, but no replication was observed in retinal pigment epithelial cells (RPEC) cultures or retinoblastoma lines (Y 79, WERI) [7].
Viruses causing retinitis in humans have been found to be members of the herpesvirus family. HSV retinitis occurs in congenital herpes simplex infection associated with herpes encephalitis [13] but can also affect healthy adults [15].

Furthermore, immunocompromised patients with AIDS or under pharmaceutical immunosuppression after solid organ transplantation are at high risk of developing CMV retinitis [8, 16]. Multiple viral infection has also been observed in these patients [33].

Clinical reports have described retinal infiltrates associated with unclear viral infections in humans [12, 22]. In most cases these lesions were self-limited and resolved within weeks without treatment. No causative agents were discovered.

Therefore the aim of our present study was to determine whether viruses other than neurotropic viruses, especially those causing common disease, are capable of infecting retinal tissue. We used RPEC in this study because epithelial cells derived from various tissues (especially kidney) are known to support the growth of several viruses of interest, e.g. herpesviruses, enteroviruses and respiratory viruses.

**Material and methods**

**Preparation of retinal pigment epithelium**

Human RPEC were isolated from freshly enucleated bulbi for corneal transplantation as previously described, with some modifications [9]. Briefly, the corneoscleral disc was first removed, followed by the lens and vitreous. The residual eye cup was sectioned with a longitudinal incision towards the optic nerve. Repeated rinsing with Dulbecco's phosphate-buffered saline (PBS), Ca\(^{2+}\) and Mg\(^{2+}\) free (Biochrom, Berlin, Germany), allowed prompt separation of the remaining vitreous and neural retina from the layer of RPEC and allowed detachment of the choroid from the sclera. The RPEC adhering to Bruch's membrane on the obtained choroidal sheets were washed with PBS and treated three times with 0.25% trypsin EDTA solution (Biochrom) for 20 min at 37 °C. The isolated cells were centrifuged at 300 g for 10 min and resuspended in RPMI 1640 (Biochrom) supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were seeded in 25-cm\(^2\) tissue culture flasks (Becton Dickinson, Plymouth, UK) for 24 h at 37 °C in a humidified atmosphere with 5% CO\(_2\). The next day, nonadherent cells were removed and fresh culture medium added. Confluent cells were trypsinized, washed with PBS and seeded into two tissue culture flasks. After two or three passages, cells were used for virus infection after seeding on Lab-Tec chamber slides (Nunc, Naperville, III).

RPEC were grown in tissue culture flasks for several passages. After seeding, cells quickly came in contact with other RPEC and began to proliferate until confluence was achieved after approximately 1 week. In the first two to three passages RPE cells still contained their typical pigmented granules. After four to six passages RPEC lost their pigmented granules and the cell proliferation rate was reduced. Figure 1 shows uninfected RPEC after 3 days of culture. For viral infection, cells were transferred onto Lab-Tec chamber slides and kept under the same culture conditions for approximately 1 week until they were confluent.

Viral infection was achieved with different viruses on early passages (one to three) as well as on later passages (four to five) of RPEC.

Virus, antibodies

HSV type 1 strain Wal has been described by Schröder et al. [35]. Human herpesvirus type 6 (HHV-6) strain GS [34], a kind gift of Dr. Salahuddin, Bethesda, Md., was propagated in interleukin-2 (IL-2)-stimulated human cord blood lymphocytes. The following viruses were obtained from the American Tissue Culture Collection (ATCC) Rockville, Md: B95-8, an Epstein-Barr virus (EBV)-transformed primate cell line releasing high titre of the virus (ATCC no. CRL 1612), CMV strain AD169 (ATCC no. VR-538), adenovirus types 1 and 7 (ATCC no. 1-VR, 7-VR), measles virus (ATCC no. 24-VR), parainfluenza virus type 1 (ATCC no. 94-VR) and coxsackie virus B3 (ATCC no. 30-VR).

RPEC cultured on Lab-Tec chamber slides (Nunc) were infected with the different viruses at high multiplicity of infection (m.o.i. >1), which was determined by standard virological methods. Cells were observed for an appropriate time until development of cytopathic effect, depending on the virus strain used, or a maximum of 3 weeks. Three different experiments were performed using pigmented RPEC (early passage) as well as unpigmented RPEC (late passage). Different cell lines, including HeLa cells, kidney cell lines, primary fibroblasts and cord blood lymphocytes, with described susceptibility or resistance against infection with the virus strains tested, served as positive and negative controls. Productive infection was confirmed by detection of viral-induced structural and nonstructural antigens in the infected RPEC by means of immunofluorescence or immunoperoxidase staining. Cells grown on the microscopic chamber slides were air-dried and subsequently fixed with ice-cold acetone. Monoclonal antibodies (mAb) or antisera against the viruses listed above were applied, diluted in PBS supplemented with 1% bovine serum albumin. They were followed by a fluorescent anti-mouse-F(ab)\(_2\)
fraction of goat immunoglobulin (Bioresearch, Kaumberg, Aus-
tria) or fluorescent anti-human IgG of rabbit immunoglobulin
(Dako, Glostrup, Denmark) and subsequently counterstained with
Evans blue (Sigma, St. Louis, Mo., USA). HSV infection was
detected with anti HSV-1 glycoprotein C antibody HC3 [19]. To
verify CMV infection, coverslips were stained with a horseradish
peroxidase-coupled goat antiserum (Biogenesis, Bournemouth,
UK) and diaminobenzidine as substrate according to standard
methods.

As a second detection method for CMV, immunofluorescence
assays to detect CMV immediate early antigen (IEA), early antigen
(EA) and late (LA) antigen were applied. Anti-CMV IEA mAb
clon E-13 was obtained from Clonatec (Biosoft, Paris, France).
MAb against CMV EA and LA were purchased from Du Pont,
(Vienna, Austria). Adenovirus infection was detected by means of
mAb Clone H60 from Clonatec. Parainfluenza virus was verified
by immunofluorescence using the Imagen Parainfluenza Virus
Group Kit (detects para influenza virus types 1, 2 and 3) from
Dako Diagnostics (Cambs., UK). Coxsackievirus and measles
virus infections were detected using high-titre positive patients'
sera. Productive EBV infection was excluded by staining with
anti-EBV gp350 mAb 72A1 (hybridoma obtained from ATCC, no.
HB168) and a mAb against the EBV major capsid protein (kindly
donated by Dr. F. Schwarzmann, University of Regensburg,
Germany). The EBV-producing cell line B95-8 was used as a positive
control. HHV-6 infection was excluded by comparing immunoflu-
orescence of the RPEC with cord blood lymphocytes freshly in-
fected with HHV-6. Antibodies used included patients' antisera
positive for HHV-6 as well as polyclonal and monoclonal antibod-
ies raised against purified HHV-6 virions (Larcher et al.,
manuscript in preparation). Infection was assumed if cells stained
with mAb and/or polyclonal antibodies in each parallel experi-
ment. Lack of infection was assumed if no infected cells could be
detected in all three parallel experiments although infection could
be shown in the control experiments described earlier. The infect-
tivity of the stocks of parainfluenza virus, adenovirus, measles
virus and coxsackievirus was controlled in the respective
susceptible cells (i.e. HeLa cells and the primate cell line Vero).
CMV laboratory strain AD169 was grown on primary fibroblast
cell culture. The lymphotropic viruses (EBV strain B95-8 and
HHV-6 strain GS) were tested by infecting peripheral blood
lymphocytes or cord blood lymphocytes stimulated with phyto-
hemagglutinin and IL-2.

Results

The following viruses were able to infect RPEC in vitro: HSV
and CMV, both belonging to the Herpesviridae, subfamily Alpha-her-
pesvirinae (HSV) and Beta-her-
pesvirinae (CMV); coxsackievirus (family Enteroviri-
daе); parainfluenza virus and measles virus (both Myx-
oviridae) and adenovirus. Infection was seen in pigment-
ed (early passage) cells as well as in later passages of
RPEC with only few pigmented granules.

No productive infection was seen with the
lymphotropic HHV-6 and EBV (subfamily Gamma-her-
pesvirinae) during 3 weeks of observation, whereas the
controls (cord blood lymphocytes) clearly became in-
fected.

Comparable to the rather unspecific viral cytopathic
effects (CPE) observed in other susceptible cell types, HSV
(Fig. 2), measles virus (Fig. 3), coxsackievirus (Fig.
4), parainfluenza virus (Fig. 5) and adenovirus (Fig. 8)
also induced a broad spectrum of CPE in RPEC. Cell
rounding was observed, as well as irregular cell shape
and translocation of pigmented granules from the periph-
ery to a condensed form around the nucleus (Figs. 2a, 4a,
8a). Cell fusion and formation of giant cells (>) and cell aggregation (→), can be seen. Bars 100 μm

Fig. 2a, b Herpes simplex virus (HSV) type 1 infection of RPEC.
Pigmented early passage (a) and unpigmented later passage (b) of
RPEC infected with HSV type 1 for 2 days. The HSV-induced
cytopathic effects such as cell rounding (△), formation of giant
cells (>) and cell aggregation (→), can be seen. Bars 100 μm
Fig. 3 Measles virus infection of RPEC. Cell rounding (Δ) and cell destruction (>) was induced by measles virus in RPEC after four days of infection. Non structured dense material derived from cell destruction is visible. Bar 100 μm

Fig. 4a, b Coxsackievirus infection of RPEC. Cytopathic effects induced by coxsackievirus infection can be achieved in early passage (a) and late passage (b) of RPEC. a Cell rounding and membrane abnormalities leading to extensive protrusions (>) can be seen. Note the peripheral location of the pigment in the uninfected cell at the top (→) and the marked condensation of pigmented granules in the infected cells (Δ). b Beginning of cell rounding (→) leading to disintegration of the RPE cell layer can be observed. Bars 100 μm

Fig. 5 Parainfluenza virus type 1 infection of RPEC. Cytopathic effects induced by parainfluenza virus infection in RPEC. Cells at different stages of cell rounding in more advanced stages of infection in the center of the figure. Cells lose contact with the culture flask. Cellular protrusions (> ) and possible syncytium formation leading to bigger cells can be seen in the center of the figure. Bar 100 μm

produced only cell rounding which rapidly turned into cytolysis (Fig. 3).

The time frame of the development of the cytopathic effects varied according to the growth characteristics of the viruses tested. As could be expected from fibroblast cultures, CMV grew much slower in RPEC than did the other viruses tested. Using CMV, only a rather small proportion of RPEC became productively infected, showing the typical “owl eye”-shaped cells with enlarged nuclei which also stained positive with CMV-specific antibodies (Fig. 7b). Despite the long period until development of the CPE (1–2 weeks) typical staining of the nuclei with a cocktail of antibodies against CMV antigens (IEA, EA, LA) could be detected 48 h after inoculation with the virus by immunofluorescence (not shown). Faster-growing viruses like HSV, coxsackievirus and adenovirus infected all cells rather rapidly, within 2 days. Parainfluenza virus and measles virus
were also able to infect the majority of cells within 2–4 days, although there were considerable differences in the amounts of virus antigen detected in early stages of infection by means of immunofluorescence (Fig. 6). Also, a small proportion of cells remained refractory to infection, suggesting different susceptibility of RPEC depending on the growth cycle.

Production of viral antigen in HSV, adenovirus and coxsackievirus was verified using the specific antibodies indicated above. No productive infection of RPEC with the two lymphotropic viruses HHV-6 and EBV could be detected even after 3 weeks of observation, and no staining in immunofluorescence was seen using antibodies against viral structural antibodies (not shown).

Discussion

The results of our present study provide evidence that different groups of viruses might be responsible for unclear retinitis. As adenoviruses, enteroviruses and paramyxoviruses are rather common pathogens frequently causing diseases in the human population, their tropism for RPEC observed in vitro could be of importance in vivo. Haematogenous spread of different viruses causing viraemia seems very likely, as the multiple lesions observed in unclear retinitis can effect both eyes simultaneously [22].

Unfortunately these in vitro findings have few applicable therapeutic consequences at present. Only herpesviruses can be treated with drugs; there are no specific drugs available to challenge the other viruses listed above.

Of all the viruses tested, HSV grew best in RPEC, which is not surprising as HSV is known to have a broad host range of susceptible cells [32].

The reason why EBV and HHV-6 did not productively infect RPEC remains unclear. The exact mechanisms of viral tropism are not known in detail for the majority of viruses and depend most likely on cellular receptors, host cell transcription factors, state of activation, cell cycle and other factors. The growth behaviour of HSV, for example, varies between proliferative epithelial infection and latent neuronal infection, depending on peculiarities of the respective cell type [32]. EBV replicates in the pharyngeal epithelium but becomes latent in lymphocytes, showing only restricted expression of viral nonstructural genes. This latent infection of lymphocytes by EBV has been described to alter the
phenotype of the cells in a way capable of inducing an immune response [23].

Therefore even an abortive infection of RPEC by viruses, in general, might provide a mechanism for induction of degenerative disease. Persistent infection of retinal cells with low-level virus replication has been observed in vitro, albeit in the absence of a specific immune response [40].

Autoimmunity to retinal antigens has been implicated in the pathogenesis of endogenous posterior uveitis and retinitis pigmentosa. Reid et al. [29] found that EBV-transformed human lymphocytes from patients with retinitis pigmentosa secreted higher levels of antiretinal antibodies than those from uveitis patients and normal controls, although all groups had low serum titres of antibody.

Many mechanisms may account for immune-mediated pathology after viral infections. Another pathway for autoimmune inflammation of the eye could be molecular mimicry between the virus and host antigens. The uveitopathogenetic site of S-antigen (a 45-kDa photoreceptor...
Fig. 9a, b Lack of infection with human herpesvirus 6 (HHV-6). Resistance of early passage (a) and late passage (b) of RPEC to infection with HHV-6 virus. No cytopathic effects can be observed after 3 weeks of incubation. Cell morphology is unchanged and shows typical distribution of granules (△). Cells are growing to confluence. In immunofluorescence (not shown) no HHV-6 antigen was detected. Bars 100 μm

Fig. 10a, b Epstein-Barr virus (EBV) fails to infect RPEC in vitro. Lack of productive infection and cytopathic effects in early (a) and late (b) passages of RPEC incubated with EBV for 3 weeks. Normal cell shape and distribution of pigmented granules can be seen (△). No evidence of any cytopathic effects is visible. In immunofluorescence (not shown) no EBV structural antigen expression was detected. Bars 100 μm

T cells have been established to be the effector cells in experimental uveitis. Fukushima and co-workers found that T lymphocytes from human T lymphotropic virus type I-immunised mice could respond not only to HTLV-I antigens but also to retinal antigens of various species, which indicates that an epitope of HTLV-I antigens is cell protein) shares a number of homologies with several viral proteins [38]. In experimental uveitis models, uveitis occurred after injection of S-antigen or with one of the sequence homologues, e.g. hepatitis B virus, Moloney murine leukaemia virus, Moloney murine sarcoma virus and baboon endogenous virus [36]. Using peptides corresponding to these regions, immunologic cross-reactivity has been found; furthermore, mAb to peptides of these regions have been shown to directly induce or augment disease in animal models [2].
cross-reactive to an epitope of retinal antigens [11]. Depletion of T lymphocyte subsets in HSV-infected animals led to the conclusion that CD8 (but not CD4) cells are protective for the contralateral retina in murine HSV-induced retinitis [42].

Another experiment was performed with the coronavirus strain JHM, a neurotropic mouse hepatitis virus, which causes lesions of the retinal pigment epithelium and the neural retina. The interphotoreceptor retinoid-binding protein (IRBP) thereby became localised abnormally in the same areas as virus-induced lesions and decreased significantly [31].

The infection initiated a series of events which led to long-term reduction and redistribution of a critical photoreceptor protein. Furthermore, B cell immunity is also involved in autoimmune disease. Neutrophil granules are protective for the contralateral retina in routine HSV-infection [44].

In summary, some forms of idiopathic retinitis could be triggered by a variety of viruses capable of causing transient retinal infection. Virus replication might lead directly to cytolysis, but also limited expression of viral antigens might alter „self“, which in further consequence could trigger immune-mediated cell destruction. Release of autoantigens from RPEC has been shown to play a role in chemically induced experimental autoimmune uveitis [20] suggesting a self-perpetuating mechanism following primary cell injury [14].

Moreover, aberrant expression of MHC class II on ciliary epithelium has been observed in uveitic eyes and experimentally induced by interferon gamma in cultured human nonpigmented ciliary epithelial cells [17]. Therefore, induction of MHC class II antigens by viral infection might contribute to mechanisms involved in the development of an autoimmune disease, as MHC class II positive cells have been proposed to play a role in the induction of autoimmune uveitis [10].

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