EVIDENCE FOR MAEDI-VISNA VIRUS IN MILK EPITHELIAL CELLS OF NATURALLY INFECTED EWES

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Abstract

Epithelial cells (MEC) were isolated from milk of four maedi-visna virus (MVV) infected and two uninfected ewes, cultured in vitro, and their epithelial nature was confirmed by immunocytochemistry with cytokeratin monoclonal antibodies. PCR analysis on MEC DNA confirmed the presence of proviral DNA in half cultures from infected animals. Expression of MVV proteins was shown both by immunocytochemistry using anti-p27 mAbs and by western blot with hyperimmune serum. Supernatants of MEC from infected ewes were showed to contain infectious viruses when assayed on the indicator goat synovial membrane cells (GSM). Furthermore, p27 protein was detected in supernatants of infected MEC suggesting active production of viral particles. No cytopathic effect was noted in MEC cultures from all infected animals; however, cocultivation of MEC with GSM promoted CPE development. These results suggested that MEC of ewes naturally infected with MVV may play an important role in sustaining virus transmission through the milk.

Key words: maedi-visna virus, milk epithelial cells, viral expression.

Maedi-visna virus (MVV) is a member of the lentivirus genus within Retroviridae family that causes interstitial pneumonia, encephalitis, arthritis, and mastitis in sheep. It has been assumed that tissue macrophages are the only target population for MVV since the virus was isolated mainly from these cells of sheep naturally and experimentally infected with this virus (3). However, there is some evidence showing that epithelial cells, including cells from the mammary gland, choroid plexus, intestine, and bronchiolar epithelium can harbour MVV genome or proteins and have the ability to sustain viral replication (5, 7, 21, 25). Since the privileged way of MVV transmission is mucosal infection of newborn lambs after ingestion of colostrum or milk from infected mothers (1, 21), a special attention should be paid to the role and significance of milk epithelial cells (MEC) in MVV transmission. It was showed that mammary epithelial cells and MEC may be effectively infected with human and monkey lentiviruses HIV-1 and SIV (6, 20) as well as with bovine leukaemia virus (4). It is known that the epithelial cells from goat oviduct and those present in goat milk were highly permissive under in vivo infection with goat lentivirus - caprine arthritis encephalitis virus (CAEV) (11, 18). However, little is known about the permissiveness of milk epithelial cells isolated from sheep milk. The aim of this work was to carry out histochemical, molecular, and virological studies in order to determine whether productive viral infection occurs in MEC isolated from ewes serologically positive for MVV.

Material and Methods

Preparation and culture of MEC. Milk samples (100 ml) were obtained from four ewes, that were serologically positive for MVV, as determined by ELISA (MVV/CAEV Serum ELISA - Institut Pourquier, France), as well as from two uninfected animals, 4 weeks post parturition. MEC were prepared according to protocol described by Mselli-Lakhal et al. (18). Briefly, milk samples were centrifuged at 500 g for 10 min, and cell pellets were resuspended in 10 ml RPMI medium with 10% FBS, seeded into 25 cm² tissue culture flasks, and incubated at 37°C overnight. Next, non-adherent cells were washed out, fresh culture medium was added, and cells were incubated for at 37°C. After 2-3 weeks of culture, foci of epithelial cells emerged and, after three serial passages, homogenous MEC cultures were obtained. A part of confluent MEC cultures were co-cultivated with goat synovial membrane cells (GSM), kindly provided by Dr Y. Chebloune (UCB, Lyon, France). The cultures were maintained in 25 cm² flasks in MEM medium with 10% foetal bovine serum,
passaged at 1:3 ratios for 2-3 passages, and stained with May-Grünwald-Giemsa for CPE examination. Supernatants from both MEC and MEC/GSM cultures with visible CPE were harvested and 10-fold dilutions were used to inoculate fresh GSM cells in 24-well plates. Infected cells were maintained in culture for 7 d and virus titres were calculated.

**Immunocytochemistry.** MEC were cultured on glass coverslips in RPMI with 10% FCS until reaching a minimum of 50% confluence. Cytokeratin epithelial cell marker and MVV-specific protein were detected on acetone-fixed MEC by immunocytochemistry, using monoclonal antibodies for cytokeratin (clone K813, Sigma) and anti-p27 MVV (1A7 clone, kindly supplied by Dr D. Gelmetti, IZ, Brescia, Italy). After 1 h incubation with primary mAbs, cells were rinsed and incubated with goat anti-mouse IgG (Fab fragment) conjugated with horseradish peroxidase (Sigma). After washing, the cells were incubated for 10 min with diaminobenzidine, rinsed in water, and then counterstained with haematoxylin.

**Detection of virus-specific proteins.** Western blot analysis of MVV-specific protein was performed as previously described by Kaba et al. (10). Briefly, confluent monolayer of cultured MEC and MEC/GSM cells, as well as supernatants of these cultures were lysed using CHAPS buffer (Sigma) and centrifuged at 10% SDS-PAGE. The proteins were blotted onto a nitrocellulose membranes (Hybond-C, Amersham). Next, the membranes were incubated with 5% skimmed dried milk in TBST buffer (20 mM Tris, 0.5 mM NaCl, pH 7.5, 0.05% Tween 20) overnight, following the incubation steps with MVV/CAEV positive serum (VLA), diluted 1:50, for 1 h, and peroxidase labelled anti-sheep IgG (Sigma), diluted 1:5,000, for 2 h. Proteins were detected using ECL Western Blotting Analysis System (Amersham). Lysate of GSM cells infected with MVV K1514 strain was used as control.

**Provirus detection.** Semi-nested PCR and nested PCR with degenerative primers were performed as previously described by Gil et al. (8) and Leroux et al. (15) using pairs of primers from gag and pol gene. The first and second rounds of PCR were performed in 34 cycles using the same conditions. DNA was extracted from cultivated MEC and MEC/GSM cells using BLOOD Mini DNA Kit (A&A Biotechnology, Poland). Amplification of a positive (GSM cells infected with MVV K1514) and negative control (without DNA) were run in parallel with all specimens. PCR products were visualised by electrophoresis on 1.4% agarose gel containing ethidium bromide in 1x TAE buffer.

**Results**

MEC were successfully isolated and cultivated from milk of four ewes, serologically positive for MVV and from two uninfected animals. The obtained monolayers contained mainly homogenous, typical epithelial cells, which in majority were positive for cytokeratin marker (Fig. 1A). Next, we tested whether MEC of naturally infected ewes are able to acquire the infection with MVV (Table 1). Immunocytochemistry with monoclonal antibody specific for p27 Gag protein was positive for two MEC cultures (Table 1, Fig. 1B, 1C). PCR analysis was performed on DNA from MEC and MEC/GSM cells using primers specific for gag and pol genes, and two of four cultures from infected ewes were positive (Fig. 2). However, PCR analysis on cocultivated MEC/GSM cells revealed one more positive culture. No amplification was obtained in cultures from the uninfected animals. To confirm that viral proteins were correctly expressed, western blot with hyperimmune serum from SRLV-infected sheep was used to detect MVV-specific proteins in both cell lysates and supernatants. The results presented in Fig. 3 demonstrate that viral proteins were correctly processed and released from both MEC and MEC/GSM cultivated cells. Little or even lack of MVV envelope glycoprotein was observed in cell lysates and supernatants; however, the p27 protein was detected in both types of cultures.

<p>| Table 1 |
| Study on milk epithelial cells of ewes infected and uninfected with MVV |</p>
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**Fig. 1.** Detection of cellular and viral proteins by immunocytochemistry. Immunocytochemistry was performed with K813 clone of mAbs specific for cytokeratin (A) and 1A7 clone of mAbs specific for p27 MVV in MEC from infected (B) and uninfected (C) ewes.

**Fig. 2.** Electrophoresis of PCR products of gag (A) and pol (B) gene amplification. Lanes: 1 – Ladder 100 bp, 2 – DNA of K1514 cells, 3-6 – DNA of MEC cultures derived from sheep no. 6, 8, 9, 20, 8-10 - DNA of MEC/GSM cultures derived from sheep no. 8, 9, 20. Lane 7 lacks any specimen.

**Fig. 3.** Detection of MVV proteins by western blot-ECL in MEC lysates (A) and supernatants (B). Lanes: 1 - K1514 cells, 2-5 - MEC cultures derived from ewes no. 6, 8, 9, 20, 6-8 - MEC/GSM cultures derived from ewes no. 8, 9, 20.

**Fig. 4.** Cytopathic effect developed in GSM cells after incubation with supernatant harvested from MEC cultures of infected (A) and uninfected (B) ewes.
The presence of Gag protein in supernatants suggests that these cells actively produced viral particles. To determine whether MEC were permissive for viral production, and whether the released virions were infectious, supernatants from MEC and MEC/GSM cells were harvested and incubated with GSM cells. Infected GSM cells after 1-3 passages developed distinguishable multinucleated cells, thus confirming the release of biologically active virions by MEC (Fig. 4).

Despite the fact that viral proteins were expressed in MEC, no cytopathic effect was noted in cultures from all infected animals. However, CPE was quite visible when MEC were cocultured with GSM cells.

All these data clearly showed that proviral DNA of MVV and viral proteins, mainly p27 Gag protein, were present in MEC isolated from naturally infected ewes. In general, addition of goat synovial membrane cells to cultivated MEC resulted in an increase in the milk epithelial cells number, or their cultures showing MVV- specific infection patterns.

**Discussion**

MVV and CAEV infect mainly cells of the monocyte-macrophage and myeloid dendritic cells lineages (19, 22). In this study we showed that the epithelial cells isolated from milk of MVV seropositive ewes were susceptible to natural MVV infection. This is not surprising since several studies based on isolated cell populations undergoing *in vitro* infection showed that epithelial cells, including MEC, can harbour lentiviral genome and support productive virus replication (6, 17, 18). Nevertheless, the *in vitro* study using MEC isolated from infected animals offered the advantage that in the case of *in vitro* infection most cell types were shown to support productive MVV replication, unlike the situation *in vivo*, in which active virus replication is highly restricted (3). This abnormal reactivity manifested by the loss of restricted replication as a result of *in vitro* culturing would be a problem when typical *in vivo* behaviour of the virus is studied. Indeed, in our study, proviral DNA, as well as viral proteins were detected in half of MEC cultures from serologically positive ewes. The lack of successful detection of viral DNA or its proteins can be related to the existence of nonproductive infections in these cells, which is typical for MVV and other lentiviruses, like HIV-1 (12, 24).

Interestingly, MVV replicated in *in vitro* cultured MEC in the absence of any cytopathic effect and its replication led to production of infectious free viral particles, as confirmed by syncytia formation in GSM cells incubated with MEC-derived supernatants. Active replication of lentiviruses in epithelial cells without the cytopathic effect was also noted in case of certain human epithelial cells infected with HIV-1 (26, 27). However, cytopathic effects were noted when MEC were cocultured with goat synovial membrane cells, which are highly permissive to lentiviral infection. The same pictures were observed with cells (TIGMEC) derived by immortalisation of mammary epithelial cells from goats naturally infected with CAEV (17). These results suggested that sheep/goat milk epithelial cells are resistant to the fusogenic activity of lentivirus envelope, or the fusion domain of the lentivirus envelope transmembrane is not well exposed at the surface of milk epithelial cells. This can be related to the usage of unusual receptor by lentiviruses to enter MEC, and up to date the nature of this receptor is unknown. Recent data have demonstrated that human MEC express HIV-1 receptor proteins CD4, CCR5, CXCR4, and galactosyl ceramide; however, no evidence was found for direct infection of MEC with HIV-1 (6).

Many studies have demonstrated that transmission of MVV can occur through ingestion of colostrum of infected ewes (9, 23). Transmission is generally attributed to lentivirus-infected macrophages; however, the presence of infected mammary and milk epithelial cells strongly suggest the involvement of these cells in virus transmission (5, 13, 14, 18). Since we showed that MEC were permissive to MVV infection we can postulate that MEC presented in colostrum of infected ewes may contribute to virus transmission by passing MVV to intestinal epithelial cells of lambs. Indeed, this pathway was demonstrated by epithelial labelling showing MVV absorption to intestinal epithelium up to 2 d after birth (21). Molecular mechanism, by which MEC harboured and transmitted MVV is unknown, but like in case of HIV-1 the vesicular transcytosis across MEC via galactosyl ceramide (GalCer) receptor may be possible (6).

High permissivity of MEC demonstrated in this study can be also contributed to relatively high frequency of mastitis developed in MVV infected ewes. Lesions developed in the mammary glands are manifested mainly by a diffuse hardening of mammary tissues, often observed after parturitions with subsequent lack of milk production (16). It was postulated that tissue macrophages would be the cells harbouring the virus; however other studies clearly showed the presence of MVV-positive cells, most likely mammary epithelial cells, in the stroma and epithelial alveolar barrier in tissue sections of the mammary glands with indurative mastitis (2). Capture and constitutive production of MVV by sheep mammary epithelial cells might trigger the lymphocyte infiltration, and in fact can accelerate the inflammatory process. Such possibility was clearly shown in human mammary epithelial cells, which endocytosed HIV-1 and facilitated viral infection of CD4+ lymphocytes (6).

In summary, findings from this study demonstrated that milk epithelial cells isolated *in vitro* from milk samples of serologically MVV-positive ewes were naturally targeted by the virus. Since MEC were permissive for MVV these results highlight the significant role of these cells in sustaining cell-associated and cell-free virus and its dissemination through milk of infected ewes.

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References


