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Extracellular vesicles in cattle infected with bovine leukaemia virus: isolation and molecular analysis

Maria Szczotka[1]✉, Magdalena Wasiak[2], Jacek Kuźmak[1]

[1]Department of Biochemistry, [2]Department of Pathology National Veterinary Research Institute, 24-100 Puławy, Poland
szczotka@piwet.pulawy.pl

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Abstract

Introduction: Exosomes are nanosized lipid bilayer membranous microvesicles, extracellularly released from a variety of mammalian cells. They mediate intercellular signalling by transporting several types of RNA, lipids and proteins and participate in the intercellular exchange of DNA, RNA, micro RNA, proteins and other components. These microvesicles are present in all body fluids in physiological and pathological conditions and reflect the state of the host organism. The aim of the study was the isolation and molecular determination of exosomes in blood and supernatant fluids of bovine dendritic cell cultures infected with bovine leukaemia virus (BLV). **Material and Methods:** Exosomes were isolated by ultracentrifugation from the blood sera, plasma and supernatant of bovine BLV-infected and uninfected control dendritic cell cultures and their presence was confirmed with scanning electron and transmission electron microscopy. Western blot analysis of the structural BLV glycoprotein 51 (Env) and protein 24 (Gag) and of the tetraspanin exosomal markers CD9, CD63 and flotillin-1 was undertaken in BLV+ and control BLV− cattle. **Results:** In exosomes of leukaemic cattle both BLV proteins and exosomal markers were detected. In healthy control animals only exosomal markers were determined. **Conclusion:** Proteins of BLV were released with exosomes and could be transferred into recipient cells as an alternative propagation route not requiring virus infection.

Keywords: bovine leukaemia virus (BLV), extracellular vesicles, exosomal markers.

Introduction

Enzootic bovine leukosis is a disease widespread in dairy herd livestock in many countries and is currently one of the most commonly reported neoplastic diseases in cattle. It is a cause of serious economic losses in cattle herds and is listed in the World Organisation for Animal Health's Terrestrial Animal Health Code (26). The causative agent responsible for the disease is bovine leukaemia virus (BLV), a member of the *Deltaretrovirus* genus in the *Retroviridae* family. It is closely related to the primate T-lymphotropic viruses types 1–5 (HTLV-1 to HTLV-5). Infection with BLV may frequently be present without clinical changes (asymptomatic), but in approximately 30% of infected cows persistent lymphocytosis was observed (4). This form of the disease is characterised by non-malignant polyclonal expansion of B-cells, and in 1–5% of infected cattle B-cell lymphoma can occur after a long latency period. Asymptomatic animals in the aleukaemic state are virus carriers and sources of infection (10, 20, 17). Bovine leukaemia virus is present in circulating peripheral blood lymphocytes, and dendritic cells (DCs) can be transmitted both horizontally and vertically by direct contact with contaminated materials such as blood, saliva and semen and by veterinarian activities (blood drawing, rectal examination, *etc*.). Vertical routes include the feeding of calves with infected colostrum and *in utero* infection (7, 14, 16). Transplacental infection is considered the predominant route of vertical transmission of bovine leukaemia virus, but how the virus crosses the blood–placenta barrier is still unclear (15).

The viraemic state is rather short and can be detected during the first days after viral infection. The detection of viral antigens is difficult because viral protein production is suppressed by cytokines, especially by interleukin (IL)-6 and IL-10 (20, 23). These investigations indicated that BLV infection caused disease when changes occurred in host factors. There is no vaccine against or treatment for bovine leukosis.

Cells of eukaryotic and prokaryotic organisms release heterologous nanoparticles 40–5,000 nm in diameter called extracellular vesicles (EVs) into the extracellular space. The vesicles are spherical and enclosed by a phospholipid bilayer membrane (18, 19, 21). Under physiological conditions, the intact bilayer structures of EVs have the capability to protect their biomolecules against degradation by ribonucleases and digestive enzymes and maintain their integrity and biological activity (11). Extracellular vesicles were first discovered in human blood by Peter Wolf in 1967 (25) as "platelet dust", and were subsequently also detected in reticulocytes and determined to be novel transfer vesicles. Extracellular vesicles can be categorised into three main groups: microvesicles, exosomes and apoptotic bodies (18, 19). They contain bioactive molecules comprising various proteins, microRNA (miRNA), messenger RNA (mRNA), and lipid compounds similar to those of the parent cells, and can affect other cells by the transfer of receptors and genetic cargo. Many authors have indicated their role in immune responses, angiogenesis, thrombosis, homeostasis, tumour development and metastasis (22, 30). The level of EVs is elevated in the course of cardiovascular diseases, viral infections, various types of neoplasm, and auto-immune, metabolic and parasitic diseases (2, 29, 30). Additionally, EVs have some cell-specific compounds and their content may be used for early diagnosis of these diseases.

Exosomes are small, lipid bilayer membranous vesicles of endocytic origin. These extracellular nanovesicles present in various body fluids are from 30 to 150 nm in diameter and are produced by the inward budding of the limiting membrane of multivesicular bodies. Exosomes carry surface and luminal proteins which are exchanged between cells (2, 3, 22). Exosomes' protein compositions and functions depend on the organs and cell types that they were excreted from. They transfer information to the target cells *via* receptor–ligand interaction, endocytosis by phagocytosis, and direct fusion with the plasma membrane. Exosomes contain heat-shock proteins (HSP), such as constitutive isoforms of HSP70 and HSP90. These proteins are involved in antigen presentation, as they can bind antigenic peptides and participate in loading peptides onto major histocompatibility complex (MHC) molecules. Class I MHC molecules are also present in exosomes from most cell types.

One of the most abundant protein families that are found in exosomes is the tetraspanins. Several members of this family, namely CD9, CD63, CD81 and CD82, are heavily present in exosomes of any cell type. Tetraspanins interact with many protein partners, including MHC molecules and integrins, which indicates that they are included in the organisation of large molecular complexes and membrane subdomains and take part in cell penetration, invasion and fusion events.

Many other compounds are present in exosomes, flotillin proteins among them. These proteins play important roles in many biological processes such as cell proliferation, apoptosis, adhesion and invasion. It was shown that flotillins are located on lipid microdomains and that the two proteins are involved in the retraction of plasma membrane vesicles. Flotillin-1 is widely expressed in the body and may have different action in different tissues and cells.

Exosomes contain proteins and nucleic acids with involvement in infection processes, because they bear mRNA and miRNA (7, 8, 9, 24, 29). It was reported that by cell-to-cell contact, exosomes can transport infectious agents and disseminate them to healthy cells, so infection is transmitted to all organs (28, 29). Neither vaccination against enzootic bovine leukosis being possible nor treatment of it being effective or economical, countering the disease must rely on detection of markers of infection with the virus to facilitate elimination of carrier animals. The aim of the study was isolation and molecular determination of exosomes in blood and supernatant fluids of bovine DC culture infected with BLV.

Material and Methods

Investigations were performed on a group of 12 Polish black and white lowland cows at age 4–6 years naturally infected with BLV. Four healthy cows at the same age as the experimental group were enrolled as negative controls. Blood samples from animals were taken in an abattoir, and for this reason the approval of the local ethics committee was not required. The supernatants from HeLa cell cultures and cultures of foetal lamb kidney permanently infected with BLV (FLK-BLV) were used as positive controls. Blood samples were drawn from the jugular vein and collected into tubes with or without ethylenediaminetetraacetic acid (EDTA)-K2 anticoagulant. White blood cells and lymphocytes were counted. A part of the blood samples with anticoagulant was centrifuged at 2,000 × *g* for 15 min at 22°C for plasma collection. The BLV status of animals was confirmed by ELISA, RT-PCR and loop-mediated isothermal amplification tests.

The methods of DC generation and cell culture were adapted from those described by Szczotka *et al.* (23). Briefly, DCs were generated from the bovine blood monocytes. The monocytes were isolated from the blood by centrifugation in a Histopaque gradient (1.077) (Sigma-Aldrich, Merck, Darmstadt, Germany). The cells from the interphase were collected, washed twice by centrifugation in autoMACs Rinsing Solution (Miltenyi Biotec, Germany), and then incubated with immunomagnetic Microbeads (Miltenyi Biotec), which were conjugated with monoclonal mouse anti-human CD14 antibody (Miltenyi Biotec). Separated cells were cultured in Roswell Park Memorial Institute-1640 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented

with IL-4, granulocyte-macrophage colony-stimulating factor and 10% foetal bovine serum (FBS) after being washed three times. Since FBS contains bovine EVs, FBS depleted of vesicles by overnight centrifugation at 120,000 × *g* was used. Supernatants of DC culture were collected and ultracentrifuged, and exosomes were isolated. The cell pellet was suspended in 1 mL of PBS and frozen at −80°C before analysis. The lysates of FLK-BLV cells were used as a positive control.

Sera, plasma, FLK-BLV lysates, and the supernatant of DC cultures were centrifuged at 4°C at low speed (1,500 × *g*) for 10 min, in a centrifugation step to eliminate dead cells and debris, and the supernatant was passed through Millipore filters of 0.22 µm pore size. Then, the next centrifugation step was performed at higher speed (20,000 × *g*) for 1 h to eliminate cell debris and larger vesicles. The final step was performed at higher speed again (100,000 × *g*) for 1 h and extracellular vesicles were precipitated by this means. The pellet was resuspended in 10 mL of phosphate-buffered saline (PBS) and again centrifuged for 5 h at 120,000 × *g* at 4°C. The supernatant was discarded and the pellet was resuspended in a small volume of PBS and stored at −80°C for further analysis. The preparation procedure ended with the protein concentration being measured in a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The presence of exosomal and viral markers was determined with the use of the Western blot (WB) method.

The presence of BLV glycoprotein gp51 in dendritic cells and the positive control FLK-BLV cell line was determined in an immunofluorescence (IF) reaction. Monoclonal anti-BLV gp51 fluorescein isothiocyanate–conjugated antibodies (VMRD, Pullman, WA, USA) were used and cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei visualisation, then the pictures were merged.

Western blot analysis. The pellet containing exosomes was resuspended in a dedicated buffer (Total Exosome RNA and Protein Isolation Kit; Invitrogen, Carlsbad, CA, USA). The protein level was measured with the NanoDrop spectrophotometer and 50 ng of protein of every sample was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis, which was run at 100 V for 3 h. Electrotransfer to nitrocellulose (Protran 0.45 NC; GE HealthCare Life Sciences/Amersham, Chalfont St. Giles, UK) was performed overnight at 20 V in standard conditions. During immunodetection, nitrocellulose membranes were blocked in 2% bovine serum albumin in PBS. Next, blots were washed with PBS-Tween buffer and incubated at room temperature for 2 h with the mouse IgG primary antibody diluted to 1:2,000 according to the manufacturer's instructions. Monoclonal antibodies for exosomal cellular markers were used as the primary antibody, namely anti-bovine CD9 (Kingfisher Biotech, St. Paul, MN, USA), anti-bovine CD63 (Bio-Rad, Hercules, CA, USA) and anti-flotillin-1 (BD Biosciences, Lexington, KY, USA). Markers of BLV were detected with the use of monoclonal anti-gp51 and anti-p24 antibodies (both from VMRD). After washing, all blots were incubated with the goat anti-mouse IgG horseradish peroxidase–conjugated secondary antibody diluted to 1:10,000 (Thermo Scientific/Pierce Biotechnology, Rockford, IL, USA) for 1 h at room temperature, then after final washing, chemiluminescence was detected with the use of Amersham ECL (enhanced chemiluminescence) reagent (Cytiva, Marlborough, MA, USA).

Scanning electron microscopy and transmission electron microscopy. A scanning electron microscope (Zeiss, Jena, Germany) and Libra 120 transmission electron microscope (Zeiss) were used for morphological examination of DCs and exosomes, which was performed according to the method described by Hanaishi *et al.* (5). Separated exosomes were embedded in 3% agarose and initially fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Then the agarose was cut into small fragments and the material was fixed for 1.5 h in a solution of 2% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4. After washing in 0.1 M phosphate buffer at pH 7.2, samples were dehydrated in graded series of ethanol, then passed through propylene oxide and embedded in epoxy resin for transmission microscopy. Ultra-thin sections were cut, stained with 2% uranyl acetate in distilled water for 15 min and then stained in lead citrate solution for 5 min. Sections were examined in the transmission electron microscope.

Results

Exosomal and viral marker analysis revealed that the morphology of monocytes took on dendritic cell characteristics after 24 h of culture *in vitro*. These cells had typical dendritic cell shapes with multiple dendrite-like processes and veils. Western blot analysis of exosomes isolated from the supernatant of cultured cells, sera and plasma of both infected and uninfected cows detected the exosomal cellular markers CD63, CD9 and flotillin-1, which indicated that the applied methods for exosome isolation were adequate and isolation was performed appropriately (Fig.1). The presence of gp51 and p24 BLV proteins was detected in exosomes isolated from the sera, plasma and supernatants of *in vitro* culture of DCs infected with BLV (Fig. 2). Confirmation of the presence of gp51 in DCs was performed by IF staining. Green fluorescence was observed in cytoplasm of DCs, and cellular nuclei stained with DAPI were blue. The two staining pictures are merged in Fig. 3. These viral proteins were absent in exosomes isolated from healthy animals. We also investigated the infectivity of blood exosomes isolated from BLV-infected cows for different human and bovine cell types (manuscript in preparation).

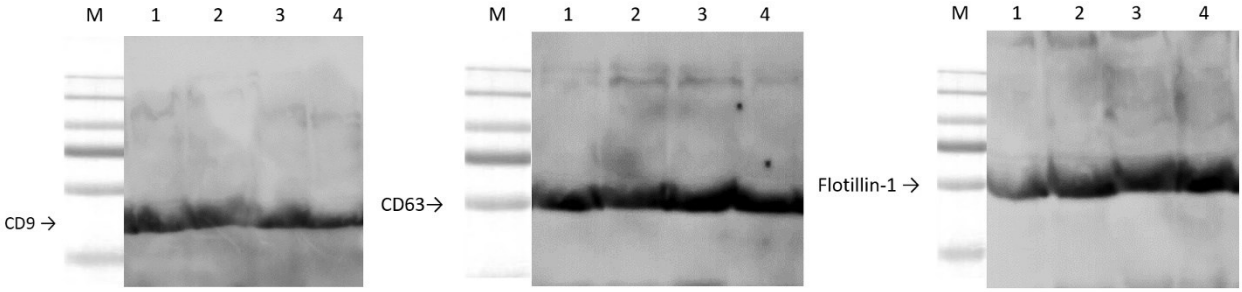

Fig. 1. The presence of CD9, CD63 and flotillin-1 exosomal markers in exosomes isolated from the blood plasma of bovine leukaemia virus–infected cows as detected by Western blot
M – Marker ladder

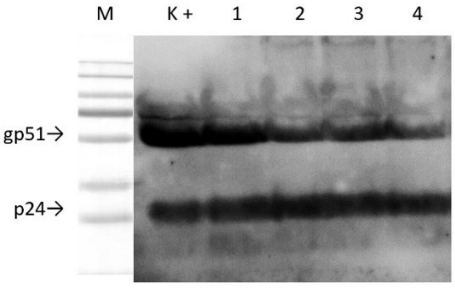

Fig. 2. The presence of glycoprotein (gp)51 and core protein (p)24 viral markers in exosomes isolated from the blood plasma of bovine leukaemia virus–infected cows as detected by Western blot
M – Marker ladder; K+ – exosomes in supernatant of permanently bovine leukaemia virus–infected foetal lamb kidney cell culture; 1–4 – exosomes isolated from the blood plasma of bovine leukaemia virus–infected cows

In exosomes isolated from the blood plasma and supernatant of *in vitro* culture of DCs from BLV-infected cows, the CD9, CD63 and flotillin-1 exosomal markers and both the p24 and gp51 viral markers were detected. The acquired images of the corresponding Western blots are presented in Figs 1 and 2.

The expression of BLV gp51 in dendritic cells generated from monocytes of BLV-infected cattle is presented in Fig. 3. The green fluorescence is visible in the whole cell, both in the veil and the processes (dendrites), which indicates the presence of BLV gp51 protein and infection with BLV. The cellular nucleus stained with DAPI is blue.

The FLK-BLV cell line was used as a positive control for the IF reaction test. Visible green fluorescence indicated the expression of BLV gp51, and blue denoted nuclei staining with DAPI.

The results of the determination of exosomal and viral markers are presented in Table 1. When supernatants of *in vitro*-cultured BLV-infected and healthy DCs were analysed, exosomal markers were detected in both groups of samples. The viral markers were detected in all samples infected with BLV.

Scanning microscopy images are presented in Figs 5, 6, 7 and 9. There are small vesicles with diameters of 1 µm between cultured dendritic cells. The cells, 10–18.8 µm in diameter, have characteristic dendrites. Figure 9 presents extracellular vesicles in the *in vitro* culture of HeLa cells.

Transmission electron microscopy images are presented in Figs 8 and 10. The visible small vesicles differ in size. Variation in vesicle size is also evident in the scanning electron microscopy images of exosomes isolated from sera, plasma and cultured DCs.

In the supernatants of leukaemic dendritic cell cultures (Figs 5, 6 and 7), extracellular vesicles 1 µm in size were detected and the cells were 18.8 µm in diameter. Similar results were found in the HeLa cell cultures (Fig. 9). Analysis in the transmission electron microscope showed the presence of vesicles 49 to 65 µm in size (Figs 8 and 10).

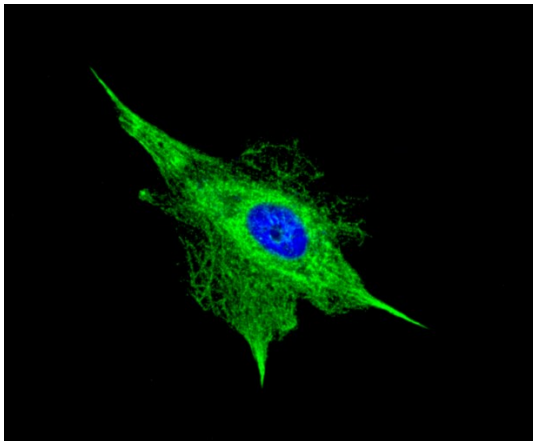

Fig. 3. Expression of bovine leukaemia virus glycoprotein 51 in dendritic cells generated from blood samples of bovine leukaemia virus–infected cattle, as visualised by an immunofluorescence reaction

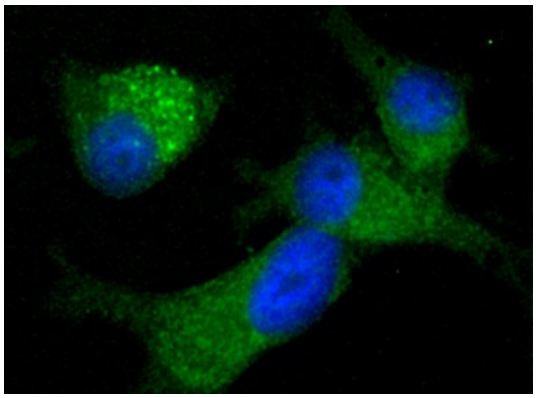

Fig. 4. Expression of bovine leukaemia virus glycoprotein 51 in a permanently bovine leukaemia virus–infected foetal lamb kidney cell line, as visualised by an immunofluorescence reaction

Table 1. The viral and exosomal markers detected by Western blot in bovine leukaemia virus (BLV)-infected cows

Origin of exosomes		BLV infection	BLV markers		Cellular markers		
			gp51	p24	CD63	CD9	flotillin-1
Positive control – supernatant of FLK-BLV culture		+	+	+	+	+	+
Supernatant of cultured *in vitro* BLV-infected bovine DCs	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+
Negative control – plasma BLV−		−	−	−	+	+	+
Positive control – lysate of FLK–BLV cells		+	+	+	+	+	+
Bovine BLV+ sera (1–7) and BLV− sera (8–11)	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+
	6	+	+	+	+	+	+
	7	+	+	+	+	+	+
	8	−	−	−	+	+	+
	9	−	−	−	+	+	+
	10	−	−	−	+	+	+
	11	−	−	−	+	+	+

gp – glycoprotein; p – core protein; FLK – foetal lamb kidney; DC – dendritic cell

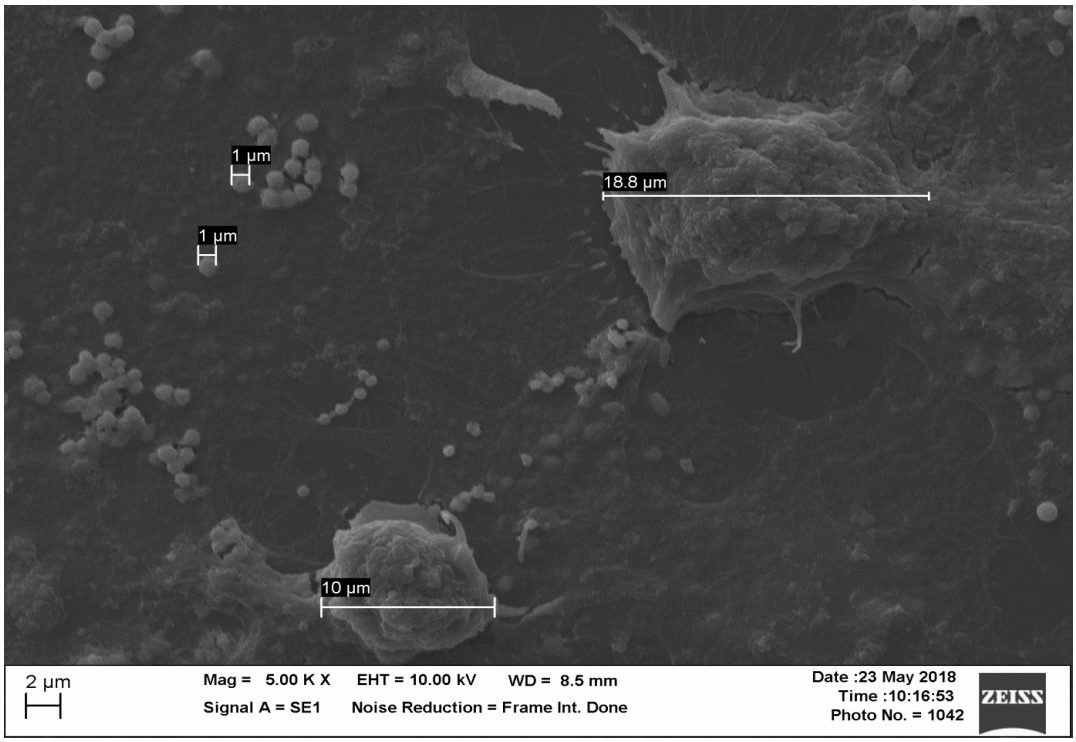

Fig. 5. Extracellular vesicles in *in vitro* culture of dendritic cells isolated from the blood of bovine leukaemia virus–infected cows seen in scanning electron microscopy
Mag – magnification; EHT – electron high tension; WD – working distance; SE1 – secondary electron 1

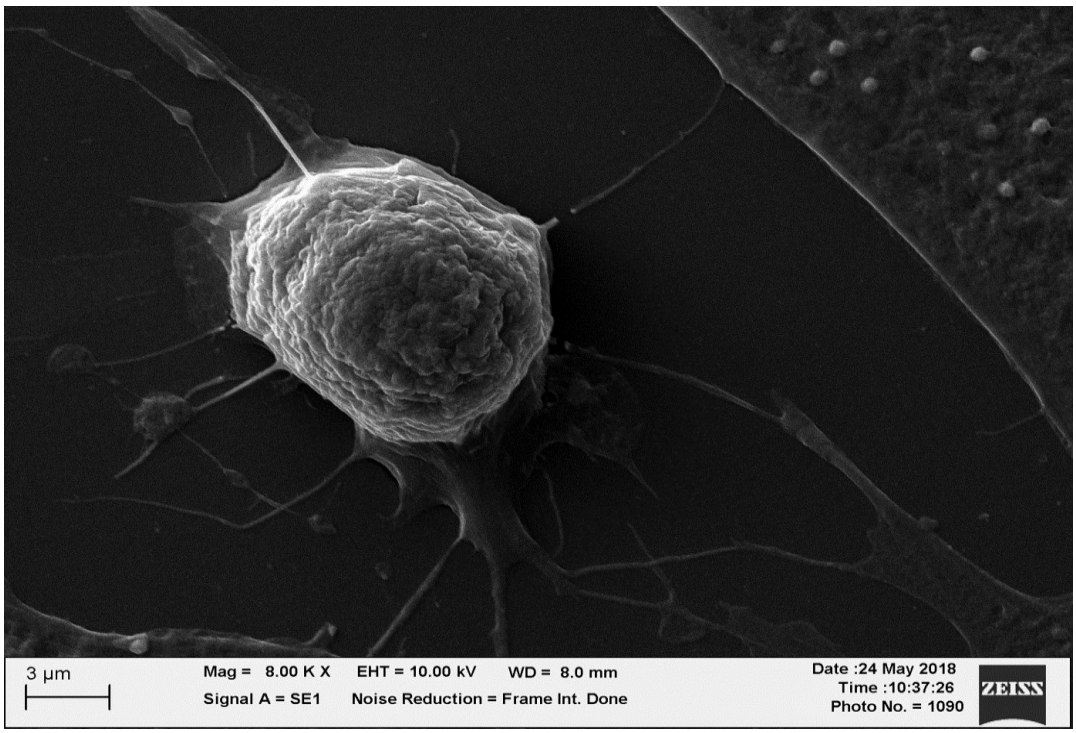

Fig. 6. Extracellular vesicles in *in vitro* culture of dendritic cells isolated from the blood of bovine leukaemia virus–infected cows seen in scanning electron microscopy
Mag – magnification; EHT – electron high tension; WD – working distance; SE1 – secondary electron 1

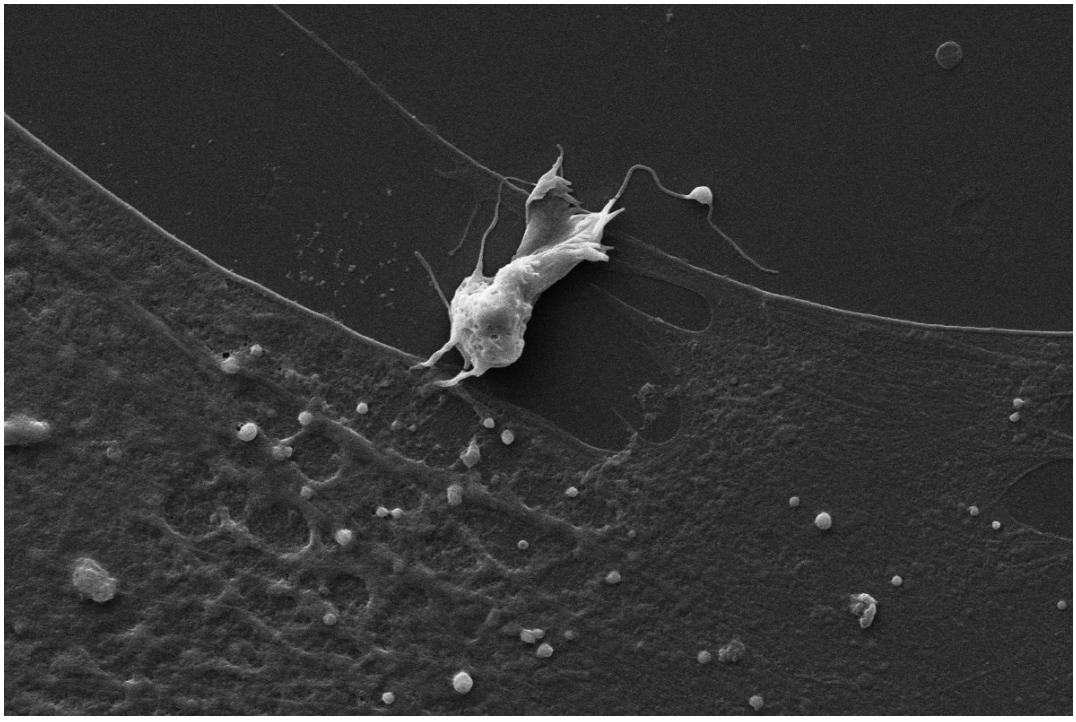

Fig. 7. Extracellular vesicles in *in vitro* culture of dendritic cells isolated from the blood of bovine leukaemia virus–infected cows seen in scanning electron microscopy. Magnification – 5.00 KX

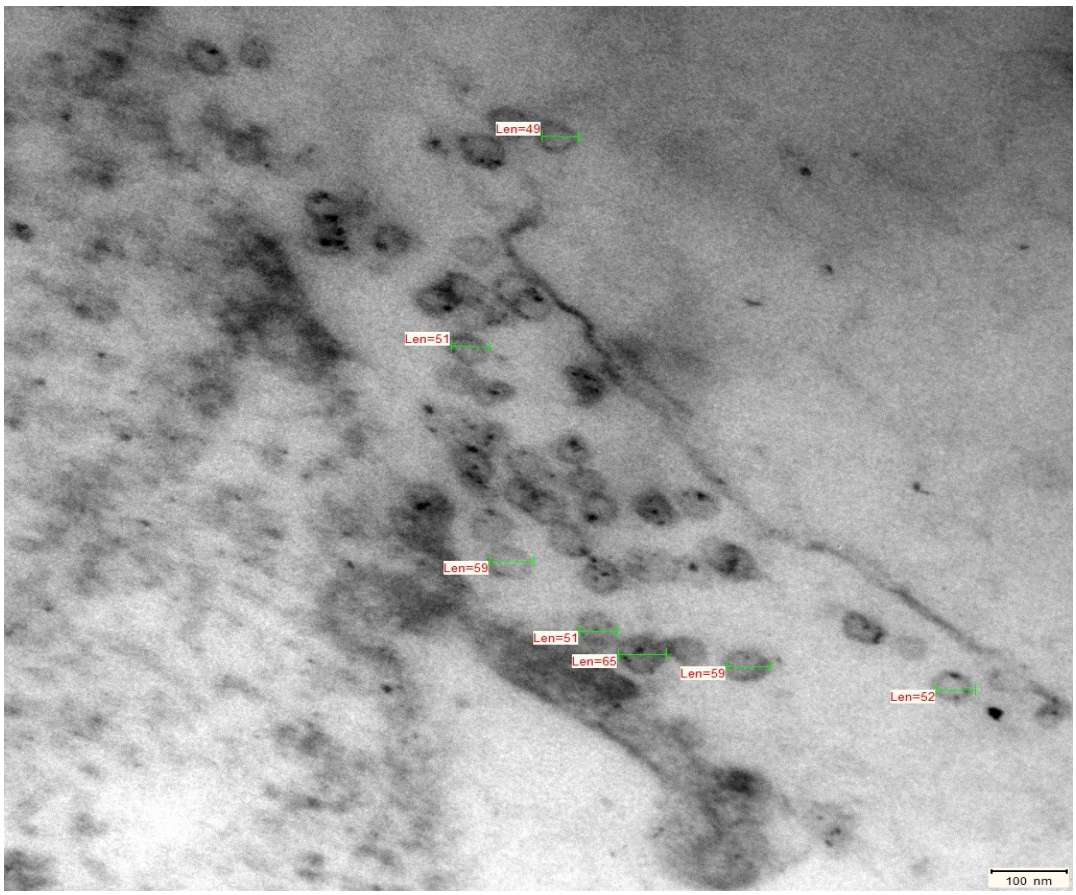

Fig. 8. Electron micrograph of exosomes in *in vitro* culture of dendritic cells seen in transmission electron microscopy
Len – size

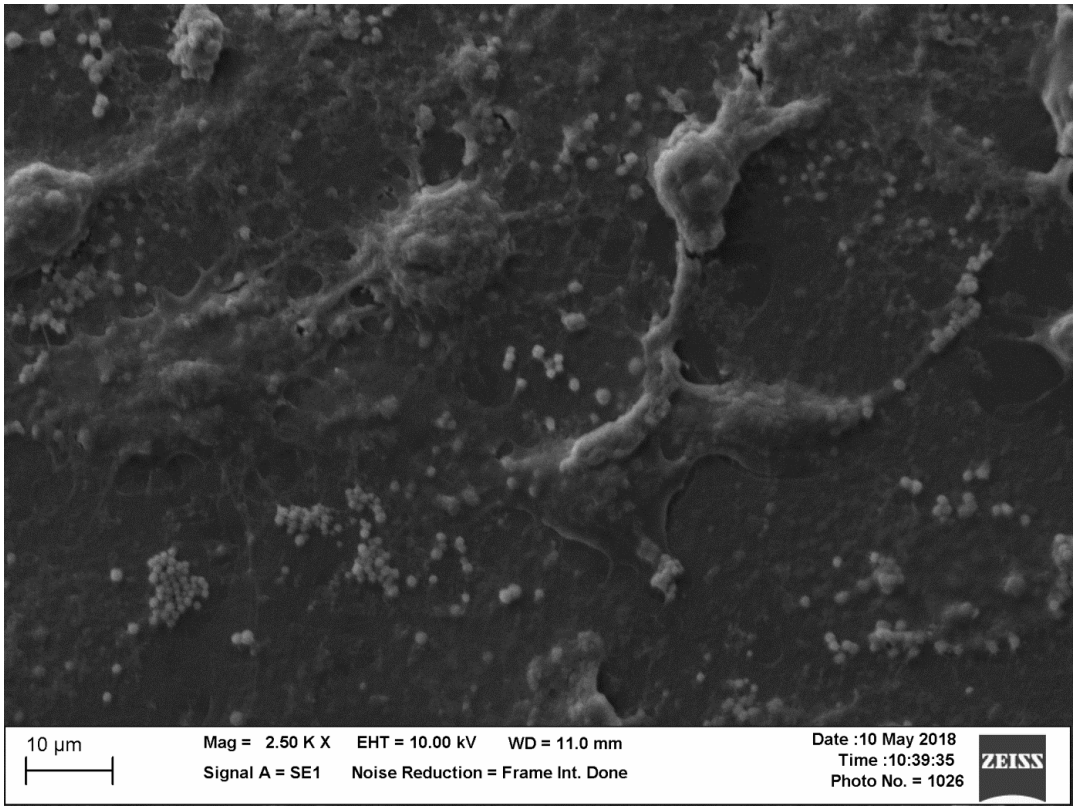

Fig. 9. Exosomes in *in vitro* culture of HeLa cells seen in scanning electron microscopy
Mag – magnification; EHT – electron high tension; WD – working distance; SE1 – secondary electron 1

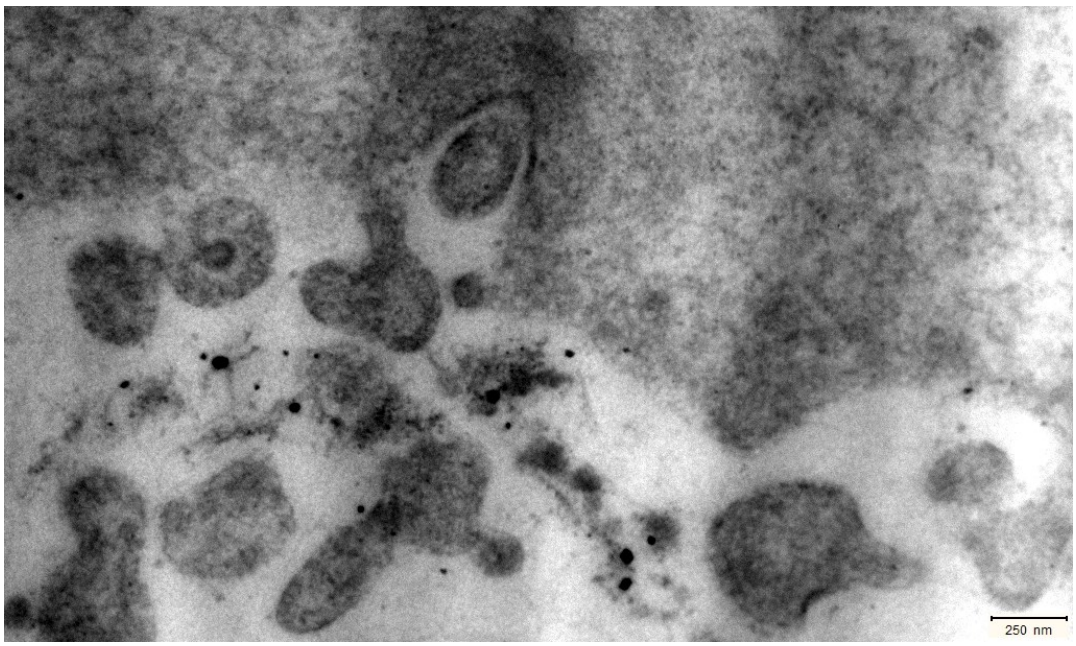

Fig. 10. Electron micrograph of exosomes in *in vitro* culture of dendritic cells isolated from the blood of bovine leukaemia virus–infected cattle seen in transmission electron microscopy

Discussion

In the current study we investigated cellular and viral markers of exosomes isolated from the blood and supernatant of cultures of DCs from BLV-infected cattle. The CD9, CD63 and flotillin-1 cellular markers were detected in these exosomes. The presence of the gp51 and p24 BLV antigens was confirmed. Yamada *et al.* (27, 28) detected viral DNA in milk samples, which confirmed the presence of BLV in milk cells of infected cows and underlined the role of milk exosomes in viral transmission. They determined the presence of viral markers in the exosomes isolated from the milk of BLV-infected cows. Different methods for exosome isolation from raw bovine milk were compared and described (3, 7, 11). Milk exosomes were characterised with typical exosomal markers: CD63, CD81, and flotillin-1 (27, 28). In exosomes isolated from milk of BLV-infected cattle, besides exosomal markers the expression of the gp51 and p24 BLV antigens was found (26, 28). Yamada *et al.* (28) investigated cell infectivity in relation to BLV gp51 and p24 in bovine milk exosomes and confirmed the presence of viral RT activity in cells, but infectivity of exosomes to cells *in vitro* was not observed.

Exosomes have great potential as liquid biopsy specimens in the diagnosis of many diseases because of their presence in most body fluids and general stability (9, 12, 18, 24, 30). Cancer-derived exosomes carry cargo reflective of genetic or signalling alterations in cancer cells and can be used as biomarkers for the early detection of neoplasia (6, 12). Exosomes contain many different proteins: both their own and proteins derived from parent cells. They are very convenient biomarkers because they have cancer-related compounds such as proteins, lipids, RNA, miRNA and DNA (18, 19, 24). They are very small, so they can easily pass through the tissue barrier and are present in various body fluids (1**,** 6, 22). Their lipid bilayer membrane protects them and their contents from degradation by enzymes that are present in circulating blood. Another factor in exosomes' diagnostic potential is specifically in the diagnosis of early-stage cancer: in humans, each millilitre of blood contains about 1×10^9 easily separated exosomes which can be analysed for indications of these diseases (6, 7, 8). Exosomes secreted by different cells are important mediators between tumour cells and stromal cells in their function of transforming information from the bone marrow microenvironment (3**,** 27, 28). Their action in cancer drug resistance and the possibility for their therapeutic application are still being investigated in many scientific centres (12). Exosomes derived from bone marrow mesenchymal stem cells play a significant role in cancer development (2, 3). Additionally, they promoted the metastatic potential of leukaemia cells (6, 13). Li *et al*. (12) developed an optimised procedure for exosome isolation from serum through the recovery of their RNA cargo and indicated the possibility for as the exploitation of exosomes as biomarkers for various diseases.

Tetraspanins are involved in the process of exosome production. In antigen-presenting cells, the functions of MHC-II molecules are regulated by their integration into the cytoplasmic membrane regions, which are enriched in the tetraspanin CD9 (3). Tetraspanins may be analysed for diagnosis of various tumours and infectious diseases. It was evident that CD63+ exosomes were significantly increased in patients with melanoma and other cancers (21, 30), and CD63 has been suggested as a protein marker of cancer. Similarly, the CD81 protein, another member of the tetraspanin family, plays an important role in cell entry by the hepatitis C virus and was demonstrated to be

significantly increased in the serum of patients with chronic hepatitis C, indicating that CD81 may be used as a marker for the diagnosis of hepatitis C viral infection (13, 22). Exosomes released by virus-infected cells contain viral components as well as components of cellular origin. It means that viruses not only transport their own products in exosomes but also exert some determining effect on the type of cellular products transported within the excretory vesicles. It was shown that exosomes released from HIV-1-infected and uninfected cells differ in their densities, which indicated that exosomes from infected cells are different from exosomes of uninfected cells (2, 21). Released exosomes can bind to neighbouring cells and travel passively through the bloodstream to very distant parts of the carrier's body, where they can induce biological changes depending on the kind of products they carry. For example, Nef is one of the HIV-1 proteins that is released within exosomes. Nef plays an important role in the activation of CD4+ T cells, and when these cells are activated, they are susceptible to HIV infection and viral replication. Some authors indicated that HIV-1 may facilitate its spread to neighbouring cells by secreting viral chemokine co-receptors CCR5 and CXCR4 in exosomes. Exosomes from HIV-1 infected cells carry several viral miRNAs (2). Another human retrovirus, HTLV-1, exports viral components with the use of an exosomal transport system (1, 12).

Exosomes serve as transporters that deliver virus receptors to target cells that make them susceptible to virus entry. Tumour-derived exosomes have been shown to promote cancer development and metastasis (11, 13). It was found that bovine milk-derived exosomes can be orally administered with the cancer chemotherapeutic paclitaxel, altering the drug's stability and toxicity (1). The important role of exosomes in cancer cell survival and proliferation was identified. On the other hand, evidence suggests that exosomes are useful in cancer treatment and may also be an effective approach in leukaemia diagnosis (6, 8). A critical attribute of tumour cells is that these cells can evade host immune surveillance. It has been reported that exosomes derived from leukaemic cells have a suppressive effect on the immune system and that as a consequence, it is possible for them to escape the immune response. The sera of patients with acute myeloblastic leukaemia were shown to contain a higher level of exosomes than the sera of healthy people (6, 18).

Since exosomes are present in most biological fluids, they are apt to be used clinically in the early diagnosis and prognosis of viral infections and diseases caused by infective microorganisms. Conventional diagnostic procedures for leukaemia have some limitations. The method based on exosome isolation from body fluids and the supernatant of dendritic cell culture is non-invasive and can be useful for early detection of haematological malignancies (8, 12). Plasma is an important source of cancer markers; its mRNA, miRNA and protein contents can be used for the early diagnosis of the disease. Research revealed that cancer cells' exosomes had different miRNA profiles to normal cells, which is important in light of the heavy involvement of miRNAs in tumorigenesis (9, 10). Exosomes contain miRNA and other genetic information and can be exploited as biomarkers (24).

Conclusion

The results of recent studies indicate the potential role of exosomal miRNAs as biomarkers. In the future, they may become effective therapeutic tools (1, 6). A significant role of exosomes is the *in vivo* delivery of drugs, miRNA and other different molecules. Exosome research related to viral infections is still in an early state and requires more studies for an understanding to be gained of exosome biology and its involvement in viral infections.

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Animal Rights Statement: No experiments on live animals were conducted and no Ethics Committee permission was required.

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