DNA damage of blood lymphocytes and neutrophils in cattle with lymphosarcoma

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ABSTRACT: The objective of the present study was to analyze the apoptotic process in peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophil leukocytes (PMN) in cows clinically affected with lymphosarcoma. Thirteen cows were studied. Of them, eight, that were referred because of inappetance, loss of body condition, diarrhoea, constipation, protrusion of third eyelid, and exophthalmia, were seropositive for bovine leukemia virus (BLV) based on a serum enzyme-linked immunosorbent assay. Other animals were apparently healthy and were used as controls. DNA damage of PBMC and PMN was assessed using the Comet assay. The results obtained showed a statistically significant difference in DNA damage between the PBMC and PMN isolated from cows infected with BLV compared to PBMC and PMN isolated from healthy cows. This is the first article to document decreased apoptosis of blood PBMC and PMN in cattle in response to BLV infection using the Comet assay.

Keywords: apoptosis; cattle; lymphocytes; lymphosarcoma; neutrophils

Enzootic bovine leukosis (EBL), also known as bovine lymphosarcoma or bovine leukaemia, is a malignant neoplastic disease of the lymphoreticular system in cattle (Johnson and Kaneene, 1992; Radostits et al., 2007). It is caused by the bovine leukaemia virus (BLV), an oncogenic type C retrovirus that integrates as a provirus into host DNA. The disease is characterized by chronic low-grade viraemia and a long latency period (Hirsh and Chung Zee, 1999). The virus is distributed worldwide, with a reservoir in persistently infected cattle. In a slaughterhouse survey, the disease was detected in 738 cases (53.9%; Dukes et al., 1982). The pathogenesis of EBL is extremely complicated. Genetic susceptibility plays an important role and is evident in the fact that so few cattle are clinically affected (Evermann et al., 1987; Hopkins et al., 1991). Leukosis is a rare manifestation of infection, with about 1% to 5% of infected cattle developing the neoplastic disease (Johnson and Kaneene, 1992). Both T-lymphocytes and B-lymphocytes may be infected with BLV; however, the resulting tumours are thought to be composed solely of B-lymphocytes (Hirsh and Chung Zee, 1999).

A number of studies have reported that deregulation of apoptosis is an important feature of bacterial- (Damle et al., 1993; Boshell et al., 1996; Haslinger et al., 2003; Park et al., 2006; Slama et al., 2009a,b,c) and virus-induced pathogenesis (Razvi and Welsh, 1995; Thomson, 1995; Bruschke et al., 1997; Dequiedt et al., 1997; Winkler et al., 1999). Viruses develop a series of strategies to either enhance or inhibit apoptosis. Among these viruses, members of the family Oncovirinae are nonacutely lymphotropic retroviruses that induce leukaemia/lymphoma after long latency periods. These viruses include BLV and human T-lymphotropic virus types 1 and 2, which are similar in genetic organization (Sagata et al., 1985; Rice et al., 1987).

A reduction in spontaneous B cell apoptosis of PBMC was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) method after experimental inoculation of BLV into a sheep (Schwartz-Cornil et

al., 1997). Inhibition of apoptosis was also detected in cultures of B-lymphocytes isolated from BLV-infected sheep using bromodeoxyuridine (BrdU) and carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling techniques (Debacq et al., 2002; Bouzar et al., 2009). In this study, apoptosis of peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophil leukocytes (PMN) was monitored in cows clinically affected with lymphosarcoma using alkaline (pH < 13) single-cell gel (SCG) electrophoresis or 0147"Comet". The technique has proven to be simple and inexpensive, can be used for small numbers of cells with relatively high sensitivity and provides a quantitative index for DNA damage (Tice et al., 2000; Endoh et al., 2002; Mohamed et al., 2011).

MATERIAL AND METHODS

Animals, history and physical examination

Thirteen cows were examined in this study. Of them, eight diseased cases (5–12 years old), weighing 420–567 kg, and referred because of inappetance, loss of body condition, diarrhoea, constipation, protrusion of the third eye lid, and exophthalmia, were used. The remaining five cows were clinically healthy and were used as controls for the Comet assay. Animals had been treated with various medications, including oral and systemic antibiotics, corticosteroids, anthelmentics, stomachics and parenteral fluid therapy. All animals were seropositive for BLV based on a serum enzyme-linked immunosorbent assay (ELISA) (Simard et al., 2000).

Isolation and preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC, lymphocytes) were isolated essentially as described previously (Nagahata et al., 1994; Higuchi and Nagahata, 2000). Briefly, to 10 ml of heparinized blood, 25 ml of phosphate buffered saline (PBS) and 10 ml of Ficoll-Conray solution (specific gravity 1.078) were added, before centrifugation at 1500 rpm/30 min/25 °C. The lymphocytes were isolated and then washed three times in PBS, with centrifugation steps at 1500 rpm/10 min/10 °C. The cells were subsequently washed with PBS until the supernatant became clear. The resulting cell population comprised < 95% lymphocytes, as determined by Wright-Giemsa staining, and < 99% of the cells were viable when assessed by trypan blue dye exclusion.

Isolation and preparation of polymorphonuclear neutrophils leukocytes

Polymorphonuclear neutrophil leukocytes (PMN, neutrophils) were isolated from heparinized blood using a Ficoll-Conray solution (specific gravity 1.078), followed by hypotonic red blood cell lysis, as previously described (Nagahata et al., 1994; Higuchi and Nagahata, 2000). Neutrophils were resuspended in Hanks' balanced salt solution (HBSS, containing Ca^{2+} and Mg^{2+}; Nissui pharmaceutical company, Japan) to a concentration of 5×10^6 cells/ml. The resulting cell population comprised < 95% neutrophils, as determined by Wright-Giemsa staining, and 99% of the cells were viable when assessed by trypan blue dye exclusion.

Evaluation of apoptosis

Apoptosis of PBMC and PMN in the blood of BLV-infected cows was evaluated based on the determination of single cell gel electrophoresis (Comet assay). Under alkaline conditions, the Comet assay of isolated PBMC and PMN was performed basically as previously reported (Endoh et al., 2002; Mohamed et al., 2011). Briefly, the isolated PBMC and PMN were embedded in 1% low-melting point agarose (Life Technologies Co., Ltd., Japan) and deposited on top of a 1% agarose base layer (Nakarai Techs Co., Ltd., Osaka, Japan) on fully frosted slides (Matsunami Glass Indust. Ltd., Tokyo, Japan). Two slides per animal were made, one for PBMC and the other for PMN. After solidification of the top layer of agarose, the slides were placed in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris-HCl, 1% Na-sarcosinate, 10% dimethyl sulphoxide and 1% Triton X-100, pH 10.0) for one hour at 4 °C in a dark room. After lysis, cell membrane and cytosol were lysed and isolated nuclei were retained in the agarose. The slides were then incubated in an electrophoretic buffer (0.3M NaOH, 1mM EDTA) for 30 min. Electrophoresis was carried out at 25 V and approximately 400 mA for 25 min at room temperature. The slides were neutralized in 0.4M Tris-HCl

solution (pH 7.5) for 20 min, stained with propidium iodide (PI), and then photographed under a fluorescent microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Images were captured with a Sony CCD camera and saved using Image Pro Plus software. Image analyzer software (Rio Grand Software) was used to quantify the different parameters of the images. Generally, 100 images were analyzed per slide. Migration length of nuclei and total length inclusive of nucleus and tail were determined, and then tail length was determined for each cell. DNA strand breaks measured by this assay are expressed as the "tail momentum" which is the product of the fraction of DNA that has exited the nucleus multiplied by the distance migrated.

Statistical analysis

Data are presented as means ± standard deviation of the mean. All statistical analyses were performed using computer software (SPSS version 17.0, Chicago, Illinois, USA). For comparisons between means of control and diseased cows, Student's t-test was used. A P value < 0.05 was considered significant.

RESULTS

Figure 1 shows DNA integrity in PBMC measured by the Comet assay, compared to control lymphocytes (0.36 ± 0.33 vs. 0.75 ± 0.29). Figure 2 shows DNA integrity in PMN measured by the Comet assay, compared to control neutrophils (0.30 ± 0.26 vs. 0.67 ± 0.21). In cows with lymphosarcoma, the decrease in DNA damage in PBMC and PMN ($P < 0.01$) was mostly evidenced by a decrease of comet momentum. When cells processed for the comet assay were examined by fluorescence microscopy, fluorescent structures corresponded to the PI-stained nuclear DNA of the PBMC and PMN cells. In undamaged cells, the DNA was tight-

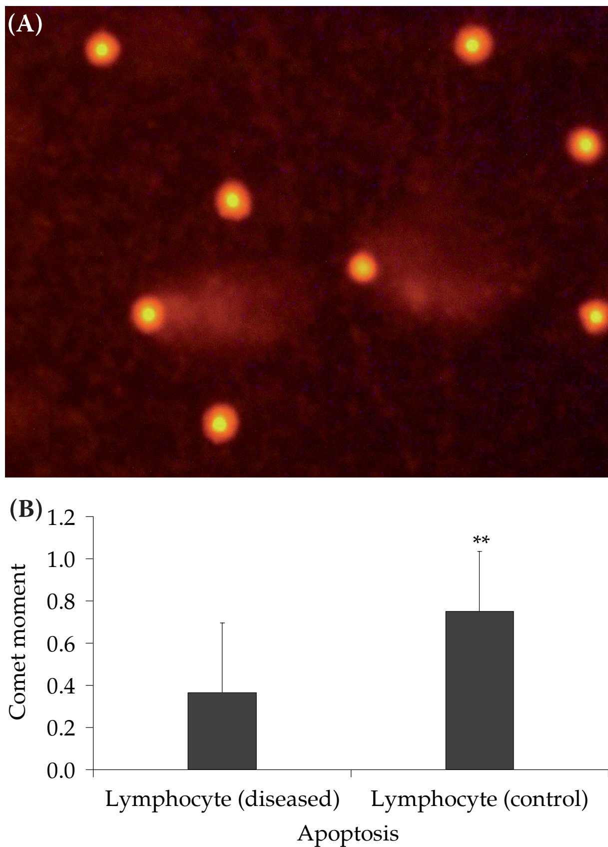

Figure 1. DNA integrity of peripheral blood mononuclear cells (PBMC) assessed by the Comet assay (A) and tail momentum of diseased (n = 8) and control (n = 5) PBMC in comet images (B). Each bar represents the mean ± SD; ** $P > 0.01$

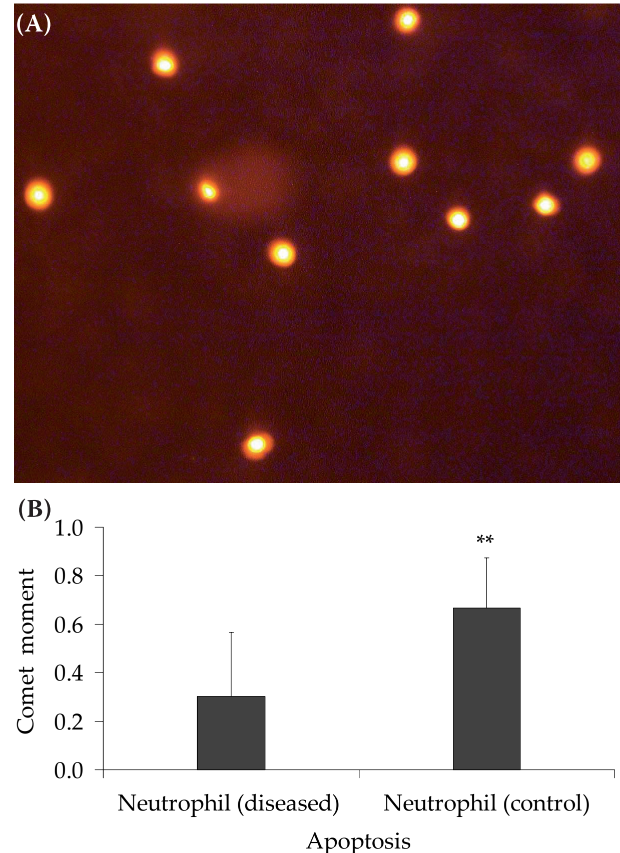

Figure 2. DNA integrity of polymorphonuclear neutrophils leukocytes (PMN) assessed by the Comet assay (A) and tail momentum of diseased (n = 8) and control (n = 5) PMN in comet images (B). Each bar represents the mean ± SD; **$P > 0.01$

ly compressed and maintained the circular disposition of the normal nucleus (Figures 1 and 2).

DISCUSSION

Single-cell gel electrophoresis is a sensitive method for measuring DNA strand breaks (Endoh et al., 2002; Mohamed et al., 2011). The clear advantage of the Comet assay over other techniques that measure DNA strand breaks is its ability to measure heterogeneity within complex populations. In the Comet assay a damaged cell takes on the appearance of a comet, with head and tail regions. A variety of geometric and densitometric parameters are provided by the image analysis software which allows an estimation of the amount of DNA in the head and tail regions and the extent of migration into the tail region. Because the tail length and density reflect the number of single-strand breaks in the DNA, the percentage of DNA in the tail provides a quantitative measure of the damaged DNA. A variety of modified Comet assays using several parameters have been developed to evaluate the extent of DNA strand breaks. The alkaline version of the Comet assay (Tice et al., 2000) primarily detects single-strand breaks in DNA. In the present study, the alkaline Comet assay was used and the extent of single-strand breaks in DNA was evaluated by the proportions of cells without tail and the average tail lengths of comet images.

Apoptosis of blood leukocytes has been studied previously using the flow cytometric technique (Van Oostveldt et al., 2001, 2002a,b; Narayanan et al., 2002; Sladek et al., 2005; Slama et al., 2006, 2007). In this study, apoptosis of PBMC and PMN isolated from cows with BLV was investigated and assessed using the Comet assay. The results obtained show a statistically significant difference in DNA damage between the PBMC and PMN isolated from cows with BLV and PBMC and PMN isolated from healthy cows ($P < 0.05$).

To the best of our knowledge, this is the first study to document decreased apoptosis of blood PBMC and PMN in cattle clinically affected by BLV infection using the Comet assay. Our data suggest that infection of lymphocytes with BLV plays an important role as an apoptosis-decreasing factor within the PBMC and PMN. The mechanism by which BLV inhibits apoptosis is unknown. However, the most straightforward explanation for this observation is that the virus synthesizes an anti-apoptotic protein, which interferes with cell death pathways. In other words, reduced susceptibility to apoptosis could prevent the destruction of an infected cell and allow the clonal expansion of the virus (Razvi and Welsh 1995; Thomson, 1995). A study is in progress to further elucidate the mechanism of apoptosis in cows clinically affected with lymphosarcoma.

Acknowledgements

The authors would like to thank Mrs. N. Peachy, English professor, for language revision.

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Received: 2010–08–28
Accepted after corrections: 2011–10–03

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