BERGAPTEN APOPTOSIS INDUCTION IN BLOOD LYMPHOCYTES OF CATTLE INFECTED WITH BOVINE LEUKAEMIA VIRUS (BLV)

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Abstract

The bergapten stimulation of lymphocytes of the cattle infected with bovine leukaemia virus resulted in the development of apoptosis within a 24-h culturing. The apoptotic process was slower in infected animals than in healthy ones and mean percentages of the apoptotic cells were 4.25, 9.75 and 13.75 after 24-, 48- and 72-h stimulation, respectively. The corresponding values in non infected animals amounted the following percentages: 8.17, 13.33 and 36.5.

Key words: bovine leukosis, lymphocytes, bergapten, apoptosis.

Apoptosis, the programmed death of cell or suicidal death, proceeds with several distinctive steps in a dying cell irrespective of the reasons. This process can be described by characteristic morphological alternations in the cell such as a specific plasma and organelle disruption into apoptotic corpses, and biochemical alterations involving DNA fragmentation (14). The apoptosis plays a key role in a proper cell differentiation and tissue homeostasis by upsetting a delicate balance between the dying and proliferating cells (12). The multistep oncogenesis induced by viruses causes a disturbed balance resulting in an increase in the number of proliferating cells from which the neoplasms cells originate (9). Thus, redressing the balance by induction of apoptosis in neoplasms cells may be an effective way for the tumour therapy (10).

The presented studies focused on the bergapten stimulation of the lymphocytes collected from the peripheral blood of healthy cattle and those infected with BLV inducing B lymphocyte proliferation in cattle and sheep (13).

Material and Methods

Blood samples. Whole blood samples (10 ml) were collected from the jugular vein of 10 dairy cattleaging 4 to 6 years. Werson II (NaCl – 8.0, KCl – 0.2, KH2PO4 – 0.2, Na2HPO4 - 1.15, EDTA – 50.0, H2O ad 1000 ml) was used as an anticoagulant in a 1 to 20 ratio.

Bergapten isolation. The bergapten preparation was obtained from Heracleum sibiricum L. fruit according to Bogucka-Kocka (1).

Isolation of lymphocytes. The whole blood diluted 1:1 with 0.9% NaCl was stratified on a 1/3 volume of Gradisol L (Polfa), centrifuged in a gradient density at 300 g for 30 min at room temperature. The lymphocyte fraction was rinsed 3 times using 0.9% NaCl. The viability of cells was examined by the trypsin blue staining.

Short term lymphocyte culturing. A suspension at a concentration of 10⁶ cells/ml was incubated in 5% CO₂ atmosphere for 72 h at 37°C. The RPMI 1640 medium with 10% calf serum, 2mM L-glutamin and antibiots (100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B) served as a growing medium. The lymphocytes were stimulated with 100 µl of 100 µM of bergapten and the apoptosis was determined 24 h, 48 h and 72 h after the stimulation. Non-stimulated lymphocytes were used as the controls.

Annexin test. The annexin V (Boehringer Mannheim) test was used to evaluate the lymphocyte
number in the early stage of apoptosis. Seventy-two hour cell cultures at a concentration of 10³ cells/mL were centrifuged at 800 rpm/10 min, then incubated for 5 min in the buffer comprising 10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, annexin V labelled with 0.65 μg/mL of FITC and iodide propidium at a concentration of 12 μg/mL. Cell morphology has examined using a BX41 Olympus fluorescence microscope. The data were processed according to the MultiScan computer program.

DNA isolation. The viral DNA samples were isolated from 500 μL of fresh whole blood using Easy Blood DNA Prep Plus kit (A&A Biotechnology, Poland).

PCR. All the examinations were conducted using two pairs of DNA primers both for the env gene and LTR gene of bovine leukaemia virus. The PCR amplification was performed in a total volume of 25 μL comprising 10 mM Tris-HCl pH 8.8 at 25°C, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton x-100, 200 μM each of the deoxynucleotide triphosphate dNTP (MBJ Fermentas), 0.5 U of the DyNaZyme II DNA polymerase (Finnzymes Oy), and 0.2 μM of each of the primers from the env gene of the BLV genome: ZM2 (5’-ctc tga tgg cta agg gca acg gc-3’) and ZM3 (5’-ctt ctc ctc cct ggg ctc cgg ca-3’) as well as two internal primers of LTR gene: L1 (5’- tgt tgg cgg cct ctt ggc cgc cgc c-3’) and L2 (5’- gtc cgg cct ctt ggc cgc cgc cgc cgc-3’) and 3 μL of BLV/DNA. The amplification temperature profile of the reaction was as follows: an initial denaturation at 95°C for 2 min, denaturation at 94°C 20 times for 30 s each, and annealing and extension at 70°C for 30 s. After the last cycle the tubes were incubated at 72°C for 7 min. The reaction mixture (5 μL) was subjected to electrophoresis on 2% agarose gel with the addition of ethidium bromide.

ELISA. The BLV (gp51) antibodies were determined in serum samples using SERELISA BLV AB (Rhône Merieux), according to the manufacturer’s recommendations.

Results

The results were shown in Tables 1 and 2, and Figs 1 to 4. Serological examinations of 10 sera by the ELISA revealed 6 positive results; 4 sera were also positive in the nested PCR (Table 1).

Table 1

<table>
<thead>
<tr>
<th>Animal number</th>
<th>OD value</th>
<th>Serological control by ELISA</th>
<th>Nested-PCR</th>
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<tbody>
<tr>
<td>1</td>
<td>0.173</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.179</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.228</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.461</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>2.068</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>2.138</td>
<td>(+)</td>
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</tr>
<tr>
<td>7</td>
<td>2.223</td>
<td>(+)</td>
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<tr>
<td>8</td>
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<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>1.958</td>
<td>(+)</td>
<td>+</td>
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Table 2

<table>
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<tr>
<th>Time of stimulation</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
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<tbody>
<tr>
<td>PCR(-)</td>
<td>8.17</td>
<td>13.33</td>
<td>36.50</td>
</tr>
<tr>
<td>PCR(+)</td>
<td>4.25</td>
<td>9.75</td>
<td>13.75</td>
</tr>
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</table>
Fig. 1. Apoptotic, living, and necrotic lymphocytes from BLV infected cattle 24 h after bergapten stimulation.

Fig. 2. Apoptotic, living, and necrotic lymphocytes from BLV infected cattle 48 h after bergapten stimulation.

Fig. 3. Apoptotic, living, and necrotic lymphocytes from BLV infected cattle 72 h after bergapten stimulation.
The OD value depending on BLV antibodies in the positive sera wranged from 1.958 to 2.223 and in the negative ones from 0.173 to 0.461. The electrophoresis of positive DNA samples showed the presence of the typical amplicon bands corresponding to 218 pb for env gene and to 165 pb for TLR gene of BLV. The size of DNA bands was the same as that of the controls obtained from FLK/BLV cells. The results of serological examinations and DNA amplification provided evidence for the expression of env and LTR genes of bovine leukemia virus in infected animals.

The results of the influence of bergapten stimulation on the apoptosis process in short term culturing of lymphocytes from both non-infected and infected with BLV cattle are presented in Figs 1, 2 and 3. As shown, the presence of apoptotic cells was observed after 24 h. When the stimulation was continued, the number of apoptotic cells increased steadily after 24 h from 7% to 11% and from 3% to 9% in the ELISA positive and negative animals, respectively. The corresponding values after 48 h ranged from 8% to 19% in positive and from 8% to 20% in negative animals. After 72 h the number of apoptotic cells in the ELISA positive animals varied from 35% to 44%, and from 10% to 38% in the ELISA negative animals.

Comparison of the results of the bergapten stimulation of the lymphocytes of infected cattle tested by nested-PCR enabled us to revealed differences between free and infected with BLV cattle. In the positive and negative animals, the number of apoptotic cells after a 24-h stimulation ranged from 3% to 6% and from 7% to 11%, respectively. Simlar results were noted after a 48-h stimulation; from 8% to 12% in the nested-PCR positive group and from 8% to 20% in the negative group. After 72 h the mean percentage of apoptotic cells ranged from 28% to 44% in nested-PCR negative animals and from 10% to 18% in the positive ones (Table 2).

The morphological and anneksin V evaluation of the apoptotic response following the stimulation of lymphocytes showed that the percentage of apoptotic cells in relation to that of the controls was lower than 5%. It should be also noted that clear differences were observed between positive and negative nested-PCR animal groups in the apoptotic response following lymphocytes stimulations (Figs 1 to 3). As it was shown, the bergapten stimulated cells in PCR negative animals demonstrated a more intensive apoptotic response in comparison to that in the positive group. The mean percentage of apoptotic lymphocytes in the nested-PCR positive animals was: 4.25, 9.75, and 13.75 after 24, 48, and 72 h of bergapten stimulation, respectively, whereas in the negative group the corresponding values were 8.17, 13.33, and 36.50.

**Discussion**

The presented study clearly indicated that nested-PCR is a very sensitive and specific method for the detection of bovine leukemia virus. The apoptosis process in the case of infected animals differed from that found in animals with negative results of nested-PCR; after a 24- 48- and 72-h stimulation of animals infected with BLV revealed a slower rate of the apoptosis process and mean percentages of apoptotic cells were 4.25, 9.75 and 13.75, whereas the negative animals demonstrated higher percentages amounting 8.17, 13.33 and 36.50, respectively. A markedly lower number of apoptotic cells in nested-PCR positive animals indicated a slow rate of the necrotic response in the lymphocytes of BLV infected cattle.

Oncogenic viruses, e.g. Epstein-Barr (EBV), SV40 virus and human papilloma virus (HPV) demonstrate similar inhibition of apoptosis. It was also found that several viruses have apoptosis suppressor genes that inhibit the cell response to infection. According to Henderson et al. (5) a latent membrane protein (LMP-1) of EBV protects the cells against the programmed death. Tewari et al. (10) submitted evidence that this protein induced the expression of A20 gene a good apoptotic suppressor.

Dequiedt et al., (3) also indicate that bovine leukemia virus modulates the cell cycle and markedly retards the lymphocyte apoptotic process in infected animals. Additionally, it was shown that the quantities of the virus in infected cells drastically diminished or even completely reduced apoptotic response. The above data indicate that viral infection promotes the cell surviving in *in vitro*.

Our finding supports the results indicating that a natural BLV may be responsible for the selective surviving of the infected lymphocytes of subpopulation B and their clonal proliferation leading to neoplasia processes (3). The inhibition of apoptosis process induced by BLV infection is not well known at present; thus, it may be suggested that a viral protein is responsible for the modulation of signal way leading to cell apoptosis.

Recently, the studies on the physico-chemical contents of several plants growing in Japan, Thailand, Sri Lanka, Uzbekistan, Belgium have provided evidence that the chemical substances isolated belong to cumarin group – piranocumarin, furanocumarin, bicumarin and
spirocumarin. These agents inhibit markedly the HIV-1 replication by the suppression of a viral enzyme reverse transcriptase. Several authors also report that some of these substances show a weak activity towards cytokine delivereation (4, 6, 7, 13, 14). It was also found that selected cumarins as the inhibitors of HIV-1 reverse transcriptase inhibits the virus replication \textit{in vitro} and protect the mouse lymphoblasts against viral cytotoxic activity. It was shown that cumarins do not inhibit the HIV-2 and the avian myeloblastosis virus (AMV) in \textit{in vitro} replication (7, 13).

The studies on the influence of cumarins on the cattle lymphocyte apoptosis enable us to understand more clearly the viral pathogenesis in the organism of infected animals, the proliferation of B lymphocytes in the organism as well as the cumarin use in the prophylaxis and therapy of retroviral infections.

\textbf{References}


