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Original article

Tumor necrosis factor-alpha (TNFα) gene polymorphism and expression of membrane-bound TNFα protein on CD11b+ and IgM+ cells in cows naturally infected with bovine leukemia virus

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Abstract

The aim of this study was to determine whether SNP at position -824 (promoter region) of the TNFα gene significantly differentiates the size of IgM+, CD5+ and CD11b+ cell subpopulations and affects the expression of membrane-bound TNFα protein (mTNFα) on these cells and their susceptibility to BLV infections.

In this study, significant differences were determined for the first time between TNFα genotypes and the percentage of cells with the CD11b+TNFα+p24+ immunophenotype. Furthermore, greater expansion of lymphocytes with the IgM+TNFα+p24+ immunophenotype was reported in cows with the G/G genotype than in A/A homozygotes. Cells with the above immunophenotype were more frequently observed in cows with persistent leukocytosis than in aleukemic cattle.

Our results suggest that polymorphism of the TNFα -824 A>G gene and mTNFα protein expression play an important role in the pathogenesis of enzootic bovine leukosis.

Key words: TNFα gene, mTNFα protein, CD5, CD11b, IgM, BLV, p24 protein

Introduction

Genetic polymorphism in the promoter region may influence the binding capacity of nuclear factors and affect the regulation of gene expression at the transcriptional level. Konnai et al. (2006) demonstrated that SNP (Single Nucleotide Polymorphism) at position -824 A>G of the TNFα gene (promoter region) can play an important role in the pathogenesis of enzootic bovine leukosis (EBL). The disease disrupts the proliferation and differentiation of B-lymphocytes. The majority of BLV-infected cattle remain in subclinical stadium (aleukaemic – AL or persistent lymphocytosis – PL form).

It is believed that similarly to humans, intra-individual variability in immune responses to retroviral

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infections in cattle can be caused by SNPs in the region of cytokine-coding genes (Tsukasaki et al. 2001, Nishimura et al. 2003). The effect of the above genetic polymorphism on BLV infections and EBL progression in cattle remains weakly investigated.

This study represents the first attempt to determine whether TNFα gene SNP at position -824 (promoter region) significantly differentiates the size of IgM+, CD5+ and CD11b+ subsets and influences the expression of membrane-bound TNFα (mTNFα) on these cells and their susceptibility to BLV. Hematological and immunophenotypic changes in BLV-infected cattle are observed mainly in B-lymphocytes with IgM and CD5 cell surface markers (Depelchin et al. 1989), whereas little is known about the role of CD11b+ cells in BLV infections and the progression of EBL in cattle.

Materials and Methods

The study covered 127 Black-and-White Polish Holstein-Friesian cows in three herds free of tuberculosis and brucellosis in north-eastern and central Poland. Blood was sampled from the mammary vein, using EDTA as the anticoagulant. Beginning from the second half of the first month of lactation, the analyzed indices were determined three times at monthly intervals in animals free of BLV and three or two times in BLV-positive cows.

Diagnosis of BLV infection

Nested PCR test

Genomic DNA was isolated from peripheral blood leukocytes using the Master Pure™ Purification Kit (Epicentre Biotechnologies, USA). The composition of the reaction mixture and the thermal profile of double amplification of the viral *env* gene were consistent with the description given by Markiewicz et al. (2003). Primers with the sequence described by Klintevall et al. (1994) (Sigma, USA) were used.

Immunofluorescence (IMF) method

PBMCs isolation and preparation for analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Histopaque 1077 from whole blood (Sigma, USA). Purified PBMCs were prepared for analysis in accordance with a previously described procedure (Kaczmarczyk et al. 2004). Isolated PBMCs suspended in buffer or in Eagle's medium with a concentration of $2x10^6$ cells/mL and minimum 98% fraction purity were used in analysis.

Identification of cells infected with BLV

Indirect IMF with the BLV3 monoclonal antibody (anti-BLVp24, IgG1, VMDR Inc. Pullman, USA) was performed according to a previously described procedure (Kaczmarczyk et al. 2008). Secondary anti-mouse IgG (H+L) antibodies with fluorochrome FITC labeling or PE were used (Invitrogen, USA). Cells expressing p24 protein were indicative of a BLV infection, whereas individuals whose lymphocytes did not show viral protein expression were regarded as infection-free. The presence of the analyzed virus was determined based on minimum 600 lymphocytes registered in the blood smear.

Polymorphism of the TNFα gene at position -824A>G

The PCR-RFLP/Sac I method was applied, as described previously (Bojarojć-Nosowicz et al. 2011).

Hematological analysis

Total leukocyte counts and lymphocyte percentages were determined with the use of a hematology analyzer, and were confirmed based on smears of blood cells stained with M-GG. The results were used to identify individuals with AL and PL. Leukocyte counts of $<12x10^9$/L and lymphocyte counts of $<8x10^9$/L were indicative of AL, whereas leukocyte counts of $>12x10^9$/L and lymphocyte counts of $8x10^9$/L were indicators of PL.

Immunophenotypic analysis

CD5+, IgM+ and CD11b+ cells, and cells coexpressing membrane-bound TNFα (mTNFα) and viral p24 protein were identified by IMF. The following primary mouse monoclonal antibodies against bovine surface epitopes were used: BIG 73A (anti-IgM) (working dilution 1.0 µg/50 µL), CACT105A (anti-CD5) (working dilution 1.0 µg/50 µL) and MM12A (anti-CD11b) (working dilution 1.0 µg/50 µL) (VMDR Inc. Pullman, USA). The epitope of the mTNFα protein was identified with mouse anti-bovine monoclonal antibody IgG2b (working dilution 0.5

μg/50 μL) (Acris GmbH, Germany). IgM, CD5 and CD11b cell surface markers were detected with the IgG (H+L) goat anti-mouse secondary antibody labeled with Cascade Blue fluorochrome (Invitrogen, USA). mTNFα protein was identified with biotinylated goat anti-mouse immunoglobulin (Dako Cytomation, Denmark) as a secondary antibody and streptavidin conjugated with Texas Red fluorochrome (Vector Lab. Inc., USA). The epitope of p24 protein was identified in accordance with a previously described procedure (Kaczmarczyk et al. 2008). The control involved procedures without the primary antibody. Smears were stored at +4°C and were analyzed under a fluorescence microscope (Axiolab-Zeiss, Germany) with the appropriate filter set, at 1000x magnification. Every analysis was performed on minimum 600 cells (subject to epitope) registered in the blood smear. The percentage of cells expressing a given epitope/epitopes was determined.

IgM+, CD5+ and CD11b+ cell surface markers on peripheral blood mononuclear cells (PBMCs) were registered in a single-color reaction. The expression of mTNFα protein on these cells was registered in a dual-color reaction. A triple-color reaction was used to detect individual PBMC subsets coexpressing mTNFα and p24 proteins. Antibodies were applied sequentially in three IMF reactions. The epitope of every cell surface marker was identified in the first reaction. The second reaction detected the p24 protein epitope. The mTNFα protein epitope was detected in the third reaction. All three IMF reactions (detection of cell surface markers, p24 and mTNFα protein epitopes) were performed on the same portion of PBMCs isolated from peripheral blood of the same individual.

Statistical analysis

The statistical analysis included data distribution fit test with Gaussian curve. Kruskal-Wallis non-parametric ANOVA and a median test for comparing specific traits were used when the distribution of the analyzed values was not consistent with normal distribution (including mathematically transformed data). Differences between groups were verified by multiple comparisons in a non-parametric test at $p<0.05$, $p<0.01$ and $p<0.001$. The results were processed in the STATISTICA 9.0 software.

Results

Analyses of TNFα -824 A>G gene polymorphism revealed the presence of all three genotypes with the following distribution: A/A – 33 individuals (26.0%), A/G – 48 cows (37.8%) and G/G – 46 individuals (36.2%). A total of 78 BLV-positive cows (group BLV+) and 49 non-infected animals (group BLV- corresponding to control) were identified. Based on the adopted classification criterion, BLV-positive cows were further subdivided into two groups of 65 individuals with AL (83.3%) and 13 cows with PL (16.7%). Leukocyte and lymphocyte counts were determined at $8.87x10^9$/L and $4.25 x10^9$/L, respectively, in BLV+AL cows, and at $20.47x10^9$/L and $15.32 x10^9$/L, respectively in BLV+PL animals. Healthy individuals were characterized by $9.45x10^9$/L leukocytes and $4.83x10^9$/L lymphocytes.

An analysis of the effect of TNFα gene polymorphism on the size of PBMC subsets in BLV-positive cows revealed significant variations between genotypes and percentages of CD11b+ and IgM+ cells (Table 1). Higher values were observed in cows with the G/G genotype than in A/A homozygotes and A/G heterozygotes ($p<0.001$). Significant differences were also reported between TNFα genotypes and the percentage of cells with CD5+, CD11b+ and IgM+ phenotypes expressing membrane-bound TNFα (mTNFα) (Table 1-1). The highest values were also found in G/G homozygotes, and the lowest – in A/A homozygotes. Furthermore, CD11b+TNFα+ and IgM+TNFα+ cells from G/G homozygotes more frequently expressed the p24 viral protein than the cells of A/A homozygotes and A/G heterozygotes (Table 1-2).

The relationship between TNFα gene polymorphism and the size of cell subpopulations were also examined in BLV-negative cows. TNFα gene polymorphism was correlated only with CD11b+TNF+ cell percentages which were higher in A/A homozygotes than in A/G heterozygotes (Table 1-1).

Moreover, the present analyses accounted for mTNFα expression, the presence of BLV and the size of blood cell subsets in both subclinical stages of EBL (Tables 2, 2-1 and 2-2). CD5+ and IgM+ cell percentages differed across groups (Table 2). Significantly higher counts of CD5+ and IgM+ cells as well as CD5+, CD11b+ and IgM+ cells expressing mTNFα (CD5+TNFα+, CD11b+TNFα+ and IgM+TNFα+ immunophenotypes) were found in cows with PL than in animals with AL ($p<0.001$), whereas the lowest CD5+TNFα+ cell percentages were reported in BLV-negative animals ($p<0.001$) (Table 2-1).

Furthermore, IgM+TNFα+ cells infected with BLV (IgM+TNFα+p24+ immunophenotype) were significantly more frequently reported in cows with PL than in individuals with AL. CD11b+TNFα+p24+ cells were detected equally often in cows with either AL or PL disease form (Table 2-2).

Table 1. Size of PBMC subpopulations in cows with different TNFα genotypes (%).

Cell subset	Cows					
	BLV+			BLV-		
	Genotypes					
	AA N=22	AG N=29	GG N=27	AA N=11	AG N=19	GG N=19
CD5+						
$\bar{x}$	11.61	11.50	12.23	12.22	11.65	11.67
SD	2.78	2.38	3.33	1.84	1.60	1.31
CD11b+						
$\bar{x}$	**4.65^{A}**	**5.62^{B}**	**7.08^{AB}**	5.97	6.28	6.33
SD	1.50	2.15	2.01	1.83	2.06	1.81
IgM+						
$\bar{x}$	**10.31^{A}**	**10.24^{B}**	**12.00^{AB}**	12.08	11.75	11.45
SD	2.95	3.02	3.87	1.53	1.58	1.20

Mean values followed by the same capital letter are significantly different at $p<0.001$.

Table 1-1. TNFα gene polymorphism and PBMC subpopulations expressing mTNFα in the analyzed cows (%).

Cell subsets expressing mTNFα	Cows					
	BLV+			BLV-		
	Genotypes					
	AA	AG	GG	AA	AG	GG
CD5+TNF+						
$\bar{x}$	**3.11^{A}**	**3.56^{b}**	**4.75^{Ab}**	3.35	3.09	3.07
SD	1.38	1.15	2.30	0.90	1.05	0.75
CD11b+TNF+						
$\bar{x}$	**2.21^{a}**	**1.97^{B}**	**3.06^{aB}**	**3.40^{f}**	**2.93^{f}**	3.07
SD	1.05	1.44	1.87	0.86	0.88	0.84
IgM+TNF+						
$\bar{x}$	**2.30^{d}**	**2.34^{e}**	**3.57^{de}**	3.27	3.41	3.05
SD	1.32	1.81	2.54	1.27	1.52	1.07

Mean values followed by the same capital or small letters are significantly different at $p<0.001$ or $p<0.05$, respectively.

Table 1-2. TNFα gene polymorphism and PBMC subpopulations expressing mTNFα and p24 viral protein (%).

Cell subsets expressing mTNFα and/or p24 viral protein	Genotypes		
	AA N=22	AG N=29	GG N=27
CD5+TNF+p24+	not investigated		
CD11b+TNF+p24+			
$\bar{x}$	**0.94^{A}**	**1.44^{B}**	**1.79^{AB}**
SD	0.77	0.87	0.96
IgM+TNF+p24+			
$\bar{x}$	**0.92^{C}**	**1.47^{d}**	**2.11^{Cd}**
SD	0.92	1.15	1.33

Mean values followed by the same capital or small letters are significantly different at $p<0.01$ or $p<0.05$, respectively.

Table 2. The size of PBMC subpopulations in the analyzed cows (%).

Cell subset	Groups of cows		
	BLV- N=49	BLV+AL N=65	BLV+PL N=13
CD5+			
$\bar{x}$	**11.79^{A}**	**11.34^{B}**	**14.35^{AB}**
SD	1.56	2.74	2.50
CD11b+			
$\bar{x}$	6.23	5.79	6.20
SD	1.91	2.21	1.90
IgM+			
$\bar{x}$	**11.71^{BD}**	**10.30^{BCD}**	**13.64^{BC}**
SD	1.44	3.29	2.43

Mean values followed by the same capital letter are significantly different at p<0.001 or p<0.01 (letter D).

Table 2-1. The size of PBMC subpopulations expressing mTNFα in the analyzed cows (%).

Cell subsets expressing mTNFα	Groups of cows		
	BLV-	BLV+AL	BLV+PL
CD5+TNF+			
$\bar{x}$	**3.14^{Aa}**	**3.64^{Aa}**	**4.87^{A}**
SD	0.89	1.72	1.86
CD11b+TNF+			
$\bar{x}$	3.08	**2.15^{B}**	**3.66^{B}**
SD	0.87	1.51	1.34
IgM+TNF+			
$\bar{x}$	**3.24^{dE}**	**2.44^{CE}**	**4.27^{Cd}**
SD	1.31	2.02	1.52

Mean values followed by the same capital or small letters are significantly different at p<0.001 or p<0.05, respectively.

Table 2-2. PBMC subpopulations expressing mTNFα and p24 viral protein (%).

Cell subsets expressing mTNFα and/or p24 viral protein	Groups of cows	
	BLV+AL	BLV+PL
CD5+TNF+p24+	not investigated	
CD11b+TNF+p24+		
$\bar{x}$	1.38	1.68
SD	0.97	0.64
IgM+TNF+p24+		
$\bar{x}$	**1.36^{D}**	**2.50^{D}**
SD	1.19	1.11

Mean values followed by the same capital letter are significantly different at p<0.001.

Discussion

Our results suggest that in BLV-positive cows, TNFα gene polymorphism induces significant variations in the size of IgM+, CD11b+ and CD5+ cell subsets expressing mTNFα. The highest cell counts were reported in individuals with the G/G genotype, and the lowest – in A/A homozygotes. In BLV-negative cows, significant differences between TNFα genotypes were observed only in CD11b+TNFα+ cells, but a reverse relationship than in BLV-positive animals was observed.

Molecule CD11b is an important receptor of complement (complement receptor type 3 – CR3) and an adhesion molecule. The results of our study suggest a relationship between TNFα -824 A>G gene poly-

morphism and the complement system where CR3 plays vital immunological functions. It also seems that in healthy cows, hematopoiesis in the myeloid-cell lineage expressing CD11b may be somewhat different in BLV-positive cows, but the role of factors regulating myeloid gene expression, including BLV, remains poorly investigated.

Interestingly, cells with the CD11b+TNFα+p24+ immunophenotype were most often ($p<0.001$) reported in animals with the G/G genotype and were least frequently observed in A/A homozygotes. Our results suggest that genotypes with two copies of the mutated gene (G/G) significantly increase the frequency of BLV-positive CD11b+TNFα+ cells. The above could imply that G/G homozygotes are more likely to have a high proviral load than A/A homozygotes where significantly lower frequency of CD11b+TNFα+p24+ cells were noted ($p<0.001$). Further work is required to validate the above observation. The presence of correlations between the TNFα -824G allele and high proviral load was reported by other authors (Konnai et al. 2006). In published studies, a higher frequency of the G/G genotype was noted in cows at clinical stages of LS than in individuals with subclinical form of AL. Recent research demonstrated the presence of correlations between selected BoLA DRB3.2 and DQA alleles vs. high proviral load (HPL) and low proviral load (LPL) (Juliarena et al. 2008, Miyasaka et al. 2013).

According to the cited authors, polymorphism of BoLA class II genes is not the only genetic factor which participates in BLV pathogenesis. They postulated the need for further research with the involvement of other genes, including TNFα genetic polymorphism (Juliarena et al. 2008).

TNFα significantly contributes to the elimination of infectious factors (Keefe et al. 1997), and it can also promote the progression of disease (Herbein and Khan 2008). Viruses can modulate or deregulate the TNF/TNFR pathway to evade the immune response and facilitate viral dissemination (Herbein and O'Brien 2000). Many viruses, including cowpox, poxvirus, EBV and HIV, encode proteins that can bind with TNFα and/or the TNFR component to inhibit TNFα functions or modulate the TNF signaling pathway (Rahman and McFadden 2006, Herbein and Khan 2008). Some viruses, such as HIV (subfamily *Lentiviridae*), use the complement system to penetrate the cell, maximize replication and spread through the host's body (Stoiber et al. 1997, Bouhlal et al. 2007).

The results of this study were interpreted in view of the hypothesis that BLV may use CD11b (CR3) molecule as co-receptor that facilitate entry into a cell. A similar mechanism is observed in human mononuclear cell lines infected with HIV. CR3 and adhesion particles facilitates HIV-1 penetration into target cells (Stoiber et al. 1997, Bajtay et al. 2004, Bouhlal et al. 2007). Similarly to humans, mTNFα could also be used by viral protein/proteins in cattle to modulate the TNF/TNFR signaling pathway and control BLV replication in CD11b+TNFα+p24+ cells. In early stages of infection, viral proteins (mainly Nef, Vpr and Tat) modulate the TNF/TNFR signaling pathway, thus enhancing HIV replication and virion production in infected CD4+ T-cells and macrophages (Herbein and Khan 2008).

In our study, significantly higher percentages of CD11b+TNFα+p24+ cells were observed in individuals with the G/G genotype than in A/A homozygotes and A/G heterozygotes, but no significant differences between cell percentages were noted in both subclinical stages of EBL. The above could indicate that CD11b+TNFα+ cells are more susceptible to BLV in G/G homozygotes and that they contribute to the initiation of infection.

Mechanisms involving TNF/TNFR pathways which promote BLV infections and contribute to EBL progression have not been studied in detail.

B lymphocytes which express surface immunoglobulin M are the main target cells for BLV (Meirom et al. 1997). This is the first study to demonstrate that the expansion of lymphocytes with the IgM+TNFα+p24+ immunophenotype is greater in cows with the G/G genotype than in A/A homozygotes. A significantly higher percentage of these cells was noted in cows with PL than in animals with AL. These data indicate a relationship between TNFα gene polymorphism, expansion of IgM+TNFα+p24+ cells and progression of EBL. A different dynamics of changes was observed in the percentage of CD11b+TNFα+p24+ cells because no significant differences were noted between cows with AL and animals with PL. The above could suggest that CR3 (molecule CD11b) plays a minor role in the progression of EBL, but it does not negate the postulated significance of CD11b and mTNFα coexpression in initial stages of infection and TNFα -824A>G gene polymorphism in the discussed process.

Our results also demonstrate that CD5+ were more frequently noted in cows with PL than in animals with AL. In group of cows with PL, CD5 molecules are constitutively dissociated from BCR, which is correlated with the protection of CD5+ cells against apoptosis induced by BCR signaling (Cantor et al. 2001). Similarly to other authors (Meirom et al. 1997, Konnai et al. 2005), we observed an expansion of IgM+ lymphocytes in BLV-positive cows and insignificant differences in the percentage of CD11b+ cells between healthy and infected animals. Most authors investigated ovine models, and in their stu-

dies, an expansion of CD11b+ cells was observed in EBL progression, whereas CD5+ cells were rarely noted in sheep (Chevalier et al. 1998).

This study demonstrated for the first time that TNFα -824 A>G gene polymorphism and mTNFα and CD11b+ coexpression on mononuclear blood cells could play an important role in initiating BLV infections. Our findings indicate that TNFα gene polymorphism and mTNFα contribute to the expansion of IgM+ cells infected with BLV, a commonly observed process in the progression of EBL. The present results seem to indicate that the polymorphism of the TNFα -824A>G gene and mTNFα plays an important role in the pathogenesis of EBL.

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