Analysis of Syk Expression in Bovine Lymphoma and Persistent Lymphocytosis Induced by Bovine Leukemia Virus

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(Received 27 May 2010/Accepted 6 August 2010/Published online in J-STAGE 20 August 2010)

ABSTRACT: Spleen tyrosine kinase (Syk) is closely related to various cell reactions. In B-cells, Syk is involved in early B-cell receptor signaling, which affects cellular survival, proliferation and differentiation. Although the kinetics of Syk mRNA and its activity are variable in different types of tumor cells, Syk may have a relation to tumor progression in many human tumors, including B-cell lymphoma/leukemia. In this study we examined whether Syk mRNA expression was changed in bovine leukemia virus (BLV)-induced persistent lymphocytosis (PL) and lymphoma. As a result, we demonstrated that the Syk mRNA expression was significantly increased in PL samples, whereas it was decreased in tumor samples. Moreover one cow, which Syk mRNA expression has been lowest among PL cattle, developed lymphoma three months later and the expression significantly decreased. These data suggest that Syk mRNA expression dynamics is closely related to BLV-induced disease.

KEY WORDS: bovine leukemia virus, lymphoma, persistent lymphocytosis, signaling, spleen tyrosine kinase.

Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase that was purified from bovine thymus and porcine spleen, and subsequently identified by immunoblotting and cloning [16, 20, 27, 32]. In B-cells, when the ligand binds to the surface immunoglobulin (Ig) of B-cell receptor (BCR), an immunoreceptor tyrosine-based activation motif (ITAM) in Ig α/β is phosphorylated by Src family kinase (SFK), and the phosphorylated ITAM binds to SYK and induces signal transduction [9, 19]. Furthermore, Syk is also required for ligand-independent signal (tonic signaling) that is initiated by ITAMs phosphorylated by SFK without ligand binding to BCR [8]. Thus, Syk plays a crucial role in the ligand-dependent/independent signal transduction that regulates proliferation and differentiation in B-cells.

In chronic lymphocytic leukemia (CLL), expression of Syk mRNA was found to be reduced in one study, but Syk was reported to be activated in another one, and also to work as an inhibitor of apoptosis [1, 2, 15]. On the other hand, Syk mRNA overexpression was detected in anaplastic lymphomakinase (ALK)-positive cells of anaplastic large cell lymphoma [28]. Moreover, Syk-dependent tonic signaling was induced in diffused large B-cell lymphoma (DLBCL). Therefore, it is suggested that Syk is closely related to oncogenesis by inducing the derangement of BCR signaling even if there is no ligand [4].

Syk expression was detected in non-hematopoietic cells as well as in hematopoietic cells [25]. Although the Syk function in non-hematopoietic cells was unclear, its expression was reduced in invasive breast cancer, malignant melanoma and gastric cancer in humans [7]. It was found that elevation of cellular motility and invasion was affected by reduced Syk expression [6, 13]. Furthermore, it was suggested that reduced Syk mRNA expression was related to poor prognosis in human breast cancer, because breast cancer patients who had reduced Syk expression significantly increased risk of metastasis compared with patients who did not [29]. Taken together, these findings suggest that the alteration of Syk expression may be closely related to oncogenesis, although the expression pattern in tumor cells differs between hematopoietic and non-hematopoietic cells.

Bovine leukemia virus (BLV) belongs to family Retroviridae genus Deltaretrovirus. Most infected cattle are asymptomatic, but approximately one-third of them suffer from persistent lymphocytosis (PL) characterized by nonmalignant polyclonal B-cell expansion and 1 to 5 percent of them develop leukemia/lymphoma. Although BLV can infect not only B-cells but also CD8+ T-cells, monocytes and granulocytes in cattle, a large number of PL and tumor cells are derived from CD5+ B-cells [24]. Although it is suggested that Tax and G4 proteins of BLV induce transformation, it is unclear what factor is related to pathogenesis progression of BLV-induced diseases [10]. Moreover, viremia is only detectable during the first two weeks of infection, and expression of the viral antigen is difficult to detect thereafter because the production of viral proteins is suppressed by cytokines such as IL-10 and -6 [10, 22, 24]. These studies suggested that the pathogenesis of BLV-induced diseases was due to not only propagation of the virus but also changes in host factors. Actually, BLV-infected B cells in PL escape apoptosis by inducing aberrant BCR signaling because BCR is dissociated from CD5 and not transferred to lipid rafts [3, 12]. Therefore, it is considered that aberrant BCR signaling is induced by BLV. In this study we investigated Syk mRNA expression by quantita-
tive RT-PCR in two BLV-induced disease stages.

MATERIALS AND METHODS

Samples: Lymph node samples from 25 cattle that developed lymphoma (T1-T25) were provided by a meat inspection center in Japan. EDTA-treated blood samples were obtained from 17 cattle that developed PL (PL1-PL17) and five healthy cattle (N1-N5) from several farms in Japan. For PL11, a blood sample was obtained again three months after the first collection. Tissue samples were stored at –80°C until use for detection of anti-BLV antibodies. Sensitivity and specificity of the test was confirmed according to the “Manual of Standards for Diagnost tests and Vaccines” issued from OIE in 2004.

Agar gel immunodiffusion test (AGID): AGID was carried out by the method described previously [17]. Briefly, the gel used for AGID consisted of 0.8% agar and 8.5% NaCl. The wells were measured 4.5 mm in diameter, and six circumferential wells were placed at a distance of 3.5 mm from the central well. The antigen was placed in the central well and the positive reference serum in 2 opposite exterior wells. The samples were placed in the remaining 4 wells. The gel diffusion plate was kept in a moisture chamber at room temperature for 48 hr, and the formed precipitation lines were observed for the presence of BLV-specific antibodies. Sensitivity and specificity of the test was confirmed according to the “Manual of Standards for Diagnost tests and Vaccines” issued from OIE in 2004.

Polymerase chain reaction (PCR): DNA was extracted from tissues and WBC using a Puregene DNA purification kit (Gentra Systems Inc., Minneapolis, MN, U.S.A.) following the manufacturer’s instructions. For PCR, a primer pair was designed was the LTR region. The oligonucleotide sequences used for the primers were 5' - GTATGAAAGATA- CTATAA-3' and 5' - TAGTTGATGCATCAGGACGC-3'. The amplification program was carried out as follows: initial incubation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 30 s. After 45 cycles, a melting curve was generated to verify the specificity of each primer. All reactions were performed in triplicate to ensure accuracy of quantitation. Expression was normalized by bovine HPRT 1 expression. All data were integrated and analyzed using iQ5 software (Bio-Rad Laboratories).

Statistical analysis: Relative Syk mRNA expression in each sample was expressed as mean ± standard deviation (S.D.), and statistically analyzed using Bonferroni’s method and Pearson’s product-moment correlation coefficient programmed in PASW statistics version 17.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Confirmation of BLV infection: BLV infections of the lymphoma-developed 25 cattle were diagnosed by PCR and confirmed that all cattle were positive. The 17 cattle with PL were confirmed to be BLV positive by both PCR and AGID. Peripheral blood leukocyte counts of these cattle were more than 15,000/μl and more than 80% of them were lymphocytes. The 5 healthy cattle were determined to be BLV negative by both PCR and AGID. Thus, a total of 42 BLV-positive cases could be defined to be samples from different stages of BLV-induced diseases (25 cattle were lymphoma and 17 were PL stage), and five BLV-negative cases were defined as control samples.

Syk mRNA expression: Syk mRNA expression was compared among tumor (T1-T25), PL (PL1-PL17) and control (N1-N5) samples. The levels of Syk mRNA expression in tumor samples varied widely, but were significantly lower than that in control samples (0.73 ± 0.44 vs. 2.73 ± 0.41 [P<0.001]) (Fig. 1). In contrast, although the levels of Syk mRNA expression in PL samples also varied widely, they were significantly higher than in control samples (3.86 ± 1.08 vs. 2.73 ± 0.41 [P=0.016]) (Fig. 1). In addition, the Syk mRNA expression was compared with the WBC count to examine the relationship between Syk and cell proliferation. However, these had no correlation (P>0.05) (Fig. 2).

Syk mRNA expression in PL11 was the lowest among the PL cattle, and the level was comparable to those in tumor samples rather than PL samples. Thus, the Syk mRNA expression and WBC counts were reexamined three months later (PL11-post). At that time, the Syk mRNA expression was significantly decreased (1.71 ± 0.14 vs. 1.03 ± 0.10 [P<0.05]) (Fig. 3) and the WBC count was dramatically increased to 130,000/μl, with a large number of atypical lymphocytes observed in blood smears.
DISCUSSION

In human leukemia and/or lymphoma, Syk seems to influence the behavior of tumor cells, but its expression is quite different among various types of neoplastic lymphocytes [2, 11, 21]. In this study, we investigated Syk mRNA expression in different stages of BLV-induced disease, PL and lymphoma. Syk mRNA expression was significantly increased in proliferated leukocytes from PL-developed cattle, whereas the expression was significantly decreased in neoplastic lymphocytes. Because the materials which used for Syk mRNA expression were different among lymphoma, PL and healthy cows, it is not easy to get a clear conclusion from the experiment. However, cDNA was synthesized from the same quantity of RNA in each material and Syk mRNA expression was relatively quantified by using HPRT 1 values. The ratios of the lymphocyte were more than 80% in cases of the peripheral blood leukocytes of PL cattle and the lymph node of lymphoma cattle. Therefore, the numbers and ratio of the lymphocytes used for Syk mRNA expression would be almost the same in each sample though the material and the clinical stage were different. Moreover, the Syk mRNA expression of PL 11, which compared using peripheral blood leukocytes, decreased with disease progression. Based on these results, it was suggested that the Syk mRNA expression was decreased in neoplastic cells, and not related to the tissues used.

In PL-developed cattle, the Syk mRNA expression was high and varied widely as compared to normal and tumor sample. The variation of the Syk mRNA expression among them may be related to the disease progression as shown in PL 11 and/or affected by host factors associated with BLV infection. The increased Syk mRNA expression may be related to PL development. In B-cells, the tandem Src homology 2 domain of Syk binds to ITAMs located at the cytoplasmic domains of BCR, and transmits intracellular signals to induce cell activation via PI3K [18]. Thus, it is suggested that the Syk in PL-developed cattle may activate...
and enhance the down-stream signal such as PI3K. It is speculated that the aberrant BCR signaling was influenced by the ITAM in gp30 of BLV envelope protein and/or factors of proliferation such as IL and cytokines altered by BLV-infection [14, 23]. However, BLV infection may be mainly affected to the Syk mRNA expression via host factors such as IL and cytokines because all B-cell is not infected with BLV in PL cattle and the BLV expression is very low in BLV-infected cell. To examine that the Syk mRNA expression relate to proliferation, we assessed the relationship between the Syk mRNA expression and WBC count in PL cattle, but there was no correlation between the increased Syk mRNA expression and WBC count. Since these cattle were kept under the different conditions, they would be affected individually by the various factors such as inflammation and stress which participated cell proliferation, and Syk mRNA expression might not have an enough effect on the WBC proliferation [7]. Actually, it is suggested that the cell proliferation rates in PL cattle increase and BLV infection affects the activation of anti-apoptosis factor such as Bcl-xL via NF-kB [10, 26]. Thus, increased Syk mRNA expression may participate as activation of anti-apoptosis factors such as Bcl-xL rather than proliferation factors.

On the other hand, compared with leukocytes from BLV-free cattle, Syk mRNA expression was significantly decreased in neoplastic lymphocytes. A decreased level of Syk mRNA was also reported in human tumor cells, and hypermethylation in the promoter region and aberrant splicing were speculated to be the causes of reduced transcription of the Syk gene [11, 30, 31]. Such mechanisms might also have act on the decrease of Syk mRNA expression in BLV-induced lymphoma. Although PL and lymphoma both had CD5+ B-cell proliferation, the Syk mRNA expression in the tumor samples was significantly decreased compared with that of the PL samples. For neoplastic lymphocytes in CLL the anti-apoptosis effect is enhanced by Syk activation, in spite of reduced Syk mRNA expression [1, 2, 15]. Therefore, it is considered that activation of SFK and/or suppression of phosphatases promotes or maintains Syk activation. In addition, other factors such as zeta-chain-associated protein 70 also enhance BCR signaling in CLL [5]. Thus, it was suggested that, although mRNA expression was decreased, Syk activation may have occurred via the mechanisms mentioned above and that Syk was responsible for oncogenesis of BLV-induced leukemia, as with DLBCL, mantle cell lymphoma and follicular lymphoma.

In conclusion, it is suggested that, although the mechanisms of the variation of Syk mRNA expression remain unclear, Syk is related to BLV-induced disease. Therefore, more investigations focusing on the kinetics and activity of Syk protein will be required to elucidate the participation of BCR signaling and the function of Syk in BLV-induced disease.

ACKNOWLEDGMENTS. The authors are grateful to Mr. Kim Barrymore for his critical reading of the manuscript and Dr. Hiroaki Nemoto for statistical advice. This study was partly supported by grants from the Ministry of Agriculture, Forestry and Fisheries, and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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45 SYK EXPRESSION IN BLV INFECTED CATTLE


