Complete suppression of viral gene expression is associated with the onset and progression of lymphoid malignancy: observations in Bovine Leukemia Virus-infected sheep

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Abstract

Background: During malignant progression, tumor cells need to acquire novel characteristics that lead to uncontrolled growth and reduced immunogenicity. In the Bovine Leukemia Virus-induced ovine leukemia model, silencing of viral gene expression has been proposed as a mechanism leading to immune evasion. However, whether proviral expression in tumors is completely suppressed in vivo was not conclusively demonstrated. Therefore, we studied viral expression in two selected experimentally-infected sheep, the virus or the disease of which had features that made it possible to distinguish tumor cells from their nontransformed counterparts.

Results: In the first animal, we observed the emergence of a genetically modified provirus simultaneously with leukemia onset. We found a Tax-mutated (TaxK303) replication-deficient provirus in the malignant B-cell clone while functional provirus (TaxE303) had been consistently monitored over the 17-month aleukemic period. In the second case, both non-transformed and transformed BLV-infected cells were present at the same time, but at distinct sites. While there was potentially-active provirus in the non-leukemic blood B-cell population, as demonstrated by ex-vivo culture and injection into naive sheep, virus expression was completely suppressed in the malignant B-cells isolated from the lymphoid tumors despite the absence of genetic alterations in the proviral genome. These observations suggest that silencing of viral genes, including the oncoprotein Tax, is associated with tumor onset.

Conclusion: Our findings suggest that silencing is critical for tumor progression and identify two distinct mechanisms-genetic and epigenetic-involved in the complete suppression of virus and Tax expression. We demonstrate that, in contrast to systems that require sustained oncogene expression, the major viral transforming protein Tax can be turned-off without reversing the transformed phenotype. We propose that suppression of viral gene expression is a contributory factor in the impairment of immune surveillance and the uncontrolled proliferation of the BLV-infected tumor cell.
Background

It is widely accepted that the majority of cancers if not all result from a combination of multiple cellular events leading to malignancy after a prolonged period of clinical latency. Alterations in the cell itself however may not be sufficient to drive full transformation and evidence has emerged that the immune system is playing a critical role in the control of cancer progression. Although the propensity of tumor cells to evade immune attack is well documented [1-3], there is little direct experimental evidence suggesting a correlation between immune evasion through virus- or oncogene-silencing and the onset of overt leukemia.

Sheep are particularly interesting as a large animal model for studying certain aspects of cancer biology. Compared to murine tumor models, information gained from large animal outbred populations such as sheep can be expected to be more informative about human malignancies [4]. Furthermore, sheep develop B-cell leukemia and lymphoma after experimental transmission of BLV, a virus belonging to the deltaretrovirus family, which encompasses HTLV-1 and -2 and STLVs [5-7]. Finally, in contrast to most rodent leukemia models in which a short mean latency precedes the aggressive acute phase, the ovine BLV-associated leukemia effectively recreates the temporal events that occur during the initiation and progression of chronic leukemia such as ATL and B-CLL in human.

In the model of BLV-induced leukemia and lymphoid tumors, viral infection and tumor progression can be monitored over time following injection with either naked proviral DNA or virus-producing cells [8,9]. BLV-infected sheep consistently develop tumors after a 6-month to 4-year period of latency. The pre-leukemic phase of infection includes the expansion of infected surface immunoglobulin M-positive (sIgM+) B-cells with proviral insertion at multiple sites, whereas a unique integration site represents the molecular signature of the provirus integrated in PBMCs isolated from S2531 demonstrated the presence of a replication-competent provirus carrying both a wild-type tax sequence (Fig. 1A, Proviral integration, EcoRI) and the YR2-derived wild-type tax (Tax_{E303}) and the YR2-derived mutated tax sequence (Tax_{K303}), consistent with our earlier studies of BLV-infected animals from the cohort to which S19 belongs [8]. In S2531, antibodies to p24, the BLV capsid protein, were detected two weeks post-inoculation and persisted over time, suggesting that productive infection with a functional wild-type virus was taking place.

Results

Sheep S2531: a case illustrating tumor-associated virus silencing by a genetic mechanism

Sheep S2531 was injected with PBMCs isolated from S19, a sheep that had been inoculated in a previous study with YR2_{LTaxSN}, a BLV-infected tumor B-cell line carrying both a silent Tax_{K303}-mutated transactivation-deficient BLV provirus and a MoMuLV-derived retroviral vector expressing a functional Tax protein [8]. In S2531, antibodies to p24, the BLV capsid protein, were detected two weeks post-inoculation and persisted over time, suggesting that productive infection with a functional wild-type virus was taking place. Sequence analysis of the BLV provirus integrated in PBMCs isolated from S2531 demonstrated the presence of a replication-competent provirus characterized by a wild-type tax sequence (Fig. 1A), identical to that initially identified in the S19 PBMCs used in the inoculum. At position 303 of the Tax protein (309 aa), we identified a glutamic acid (E) resulting from a A_{3149} to C_{3149} transition which was shown to originate from homologous recombination between the transduced LTaxSN vector-derived wild-type tax (Tax_{E303}) and the YR2-derived mutated tax sequence (Tax_{K303}), consistent with our earlier studies of BLV-infected animals from the cohort to which S19 belongs [8]. In S2531, the Tax_{K303} replication-competent provirus was identified throughout the 17-month aleukemic period, characterized by normal WBC counts and a polyclonal integration pattern of the provirus, the hallmark of a non-transformed BLV-infected B-cell population (Fig. 1A, Proviral integration, EcoRI). S2531 developed a fatal B-cell leukemia as well as lym-
phoma eighteen months post-infection. This acute phase was characterized by the development of localized B-lymphoid tumors, as well as increasing WBC counts up to 68,900/mm³, a significantly increased virus load resulting from the proliferation of the malignant B-cell clone (Fig. 1A, Viral load Sac I) and a monoclonal integration pattern of the provirus in both the leukemic PBMCs and the lymphoid tumors. Sequence analysis revealed that, in contrast to the observations with PBMCs isolated at the aleukemic stage, the provirus identified in the malignant B-cell clone was a TaxK303-mutated replication-deficient provirus carrying an A at position 8149 (Fig. 1A, red arrows).

Expression vectors for Tax2531 were then constructed by exchanging the wild-type tax sequence in pSGTax with the PCR-amplified tax DNA from either pre-leukemic (position 8149 = G) or leukemic (position 8149 = A) S2531 samples respectively. HeLa cells were co-transfected with each pSGTax2531 construct together with the pLTRLuc reporter plasmid containing the firefly luciferase gene under the control of the BLV promoter as previously described [19]. Luciferase activities examined 42 hours post-transfection of pSGTax2531 constructs from samples 17-months post-inoculation were not significantly different from background levels generated by the control vector pSGc, confirming the transactivation-deficient phenotype associated with the genetic change observed in the tumor-derived proviral tax. As expected, constructs expressing tax sequences isolated from earlier samples, before the onset of leukemia, were consistently positive (Fig. 1A,B). Furthermore, two naïve sheep injected with the cloned S2531 proviral DNA isolated from leukemic cells failed to seroconvert and BLV-specific PCR was consistently negative, conclusively demonstrating that the cell lines were representative of the parental tumors (Fig. 2C). Whereas the lymphoma-derived CL267-1, -2, -3 cell cultures CL267-1, -2, -3 cell lines were derived from these cells, displayed the same monoclonal integration pattern, suggesting that the cell lines were representative of the parental tumors (Fig. 2C). Whereas the lymphoma-derived CL267-1, -2, -3 cell lines were established from fresh L267-1, -2 and -3 cells in the absence of cytokines, culture of BL267 cells in similar conditions did not result in the outgrowth of transformed B-cells. Because cytokine-independent growth is a characteristic of B-cell transformation [12], our data strongly suggest that the blood-derived BLV-infected cells from S267 were not transformed.

B-cells freshly isolated from non-leukemic BLV-infected sheep spontaneously express viral proteins including Tax, whereas it is expected, if our hypothesis is correct, that tumor cells and the cell lines derived from these tumors harbor a silent provirus [8,15]. Using RT-PCR, we could not detect transcriptional activity in either the freshly iso-
Follow-up of sheep S2531: silencing occurs simultaneously with the onset of leukemia. (A) Blood samples were collected from S2531 at regular time intervals over a 18-month period from the time of inoculation to the leukemic stage and examined for several parameters. WBC counts per mm$^3$ are indicated. Provirus load and integration were examined by Southern blot hybridization of SacI- and EcoRI-digests respectively, showing increasing provirus load and the progression from polyclonal to monoclonal integration as leukemia develops. The nucleotide sequence of the 3′ end of the proviral tax DNA is illustrated by a polyacrylamide gel autoradiography of dideoxynucleotide sequenced PCR-amplified DNA. Boxes highlight nucleotides at positions 8149, 8150 and 8151 of the BLV sequence [29]. Arrows indicate the nucleotide identified at position 8149: a G at pre-leukemic stages (yellow arrow); a G to A transition at the time of the first documented WBC increase (17-month post-infection, red arrow). The resulting amino acid at position 303 of the corresponding Tax proteins is shown below.

The transactivation potential of the putative S2531 proviral Tax proteins were examined in a luciferase reporter assay following co-transfection of HeLa cells with the pSGTax2531 expression vectors containing tax sequences cloned from S2531 PBMCs collected at different times post-infection and the reporter plasmid pLTR-Luc as detailed in B. “+” indicates a luciferase activity equivalent to that resulting from transfection with the wild-type pSGTax; “−” indicates the background level activity similar to that obtained when the empty expression vector pSG5 is co-transfected with pLTR-Luc. (B) Luciferase assay reflecting the transactivation potential of a selection of four S2531-derived tax sequences. Each pSGTax2531 construct containing the different S2531-derived tax sequences downstream of the CMV promoter was used in HeLa co-transfection with pLTR-Luc which expresses the firefly luciferase under the control of the BLV-LTR promoter. Luciferase activities were measured in cell lysates 42 h posttransfection and were normalized to protein concentrations as previously described [19]. Results are represented as histograms indicating basal luciferase activities (arbitrary units). pSGTax2531–6 and pSGTax2531–14 contain sequences amplified from PBMCs isolated during the aleukemic stage, 6 and 14 months post-inoculation respectively; pSGTax2531–18 contains tax sequences from leukemic PBMC isolated 18 months post-inoculation, and the pSGTax2531-tum construct resulted from the insertion of lymphoma-derived tax sequences collected 18 months post-infection. pSGc is the empty control vector. Values represent the means of the results of triplicate samples. The results from a representative experiment of four independent experiments are shown.
Sheep S267: non-transformed blood-derived B-cells carry a potentially active provirus while virus and Tax expression are completely suppressed in the co-existing malignant lymphoma B-cells. (A) Diagram of the BLV L267 provirus and major transcripts. The two LTRs and the gag, pro, pol, env, tax, and rex genes are represented. Vertical arrows indicate restriction sites in the L267 provirus: S, SacI; E, EcoRI. The position and direction of the PCR primers are indicated on the provirus map. The horizontal bar indicates the 8.4 kb-long region that was used as probe. Double lines represent the sequenced regions. The genomic, env, and tax/rex transcripts are represented below. Alternatively spliced RNAs are not shown. The translation products of the singly- and doubly-spliced transcripts and the positions of the RT-PCR primers are indicated. (B) Southern blot analysis following hybridization with a full-length BLV probe of SacI-digested DNA isolated from blood (BL267) and lymphoma (L267-1, -2 and -3) cells collected from S267 twenty-nine months post-infection. SacI is indicative of the proviral load (upper row). Southern blot analysis of EcoRI-digested DNA indicates the presence of a single monoclonally-integrated provirus for all three lymphoma (L267) whereas the blood-derived BL267 cells display a polyclonal integration pattern (middle and lower panels). EcoRI-cleaved DNA generates two virus-host junction fragments for each integrated L267 provirus as illustrated in the diagram. Shown here in each lane are the fragments containing the 5’ flanking genomic region. (C) Southern blot analysis of EcoRI-digested DNA isolated from the lymphoma (L267-1, -2, -3) and the cell lines derived from each of these lymphoma (CL267) cultured for four weeks. (D) RT-PCR analysis of RNA isolated from lymphoma-derived cell lines (CL267), 24 h-cultured blood-derived lymphocytes (BL267-24 h), fresh lymphoma (L267) and freshly isolated blood-derived lymphocytes (BL267). EnvA/Tax2 primers for the detection of the doubly-spliced tax/rex RNA were used. In the controls YR2 and YR2LTaxSN, provirus is silent and active respectively. (E) PCR analysis using BLV tax-specific primer pair Tax1/Tax2 of DNA isolated from sheep inoculated with the various S267-isolated B-cell populations: six sheep were inoculated using either cultured (CL267) or fresh (L267) transformed B-cells, two sheep were injected with nontransformed PBMCs (BL267).
Table 1: Characterization of PBMC- and lymphoma-derived B-cells isolated from sheep S267

| Cells isolated from:          | Blood                        | Lymphoma                      |
|-------------------------------|------------------------------|-------------------------------|
| provirus integration          | polyclonal                   | monoclonal                   |
| cytokine-independent growth/capacity to derive cell lines | -                            | +                             |
| viral expression              | -                            | +                             |
| provirus sequence             | wild-type                    | wild-type                    |
| in vivo infectious potential  | +                            | -                             |

Discussion

Using the BLV-associated ovine model of leukemia and based on the observations in two experimental sheep, we provide evidence for the role of virus and oncogene silencing as an important step in the onset of lymphoid malignancy. In the first animal, S2531, we identified a correlation between the genetic modification of the proviral structure and the emergence of leukemia. We found a Tax-mutated (TaxK303) replication-deficient provirus integrated into the genome of the malignant B-cell clone while recombinant functional provirus (TaxE303) had been consistently monitored over the aleukemic period. Although sequencing of individual tax clones identified the presence of a replication-deficient proviral form in the inoculum, our data provide no clues as to how this provirus might persist in the infected host. It will be important to sort out from our future studies whether the TaxK303 defective provirus found at the time of leukemia development in S2531 was already present in the pre-tumoral clone early after infection. A study is ongoing to answer this question, based on a BLV-specific inverse PCR technique for the detection of tumor-specific integration sites developed by Moules et al. [24]. Using this method, BLV-positive pre-malignant clones are detectable as early as two weeks after virus exposure. Whatever the mechanism responsible for this genetic modification, our observations suggest that switching off expression of Tax, the essential contributor to the oncogenic potential of BLV, is linked with the onset of acute leukemia. We propose that in this particular case, the mechanism by which the immune system destroys developing malignancies is evaded by the malignant cell by reducing its intrinsic immunogenicity, possibly through recombination-mediated virus silencing. In the second case, S267, both non-transformed and transformed BLV-infected cells were present at the same time, but at clearly distinct sites. While there was potentially-active provirus in the non-leukemic blood B-cell population, as demonstrated by ex-vivo culture and injection into naïve recipients, virus expression was completely suppressed in the malignant B-cells isolated from the lymphoid tumors despite the absence of genetic alterations in the proviral genome. This independent observation reinforces our previous conclusion and suggests that besides genetic alterations, epigenetic mechanisms might be involved in tumor-associated virus latency.

Sheep infected by BLV mount a strong immune response to viral antigens. Active killing of infected cells might play a decisive role in limiting BLV gene expression, but seems unable to prevent – or perhaps paradoxically favors – the development of a malignant clone harboring a silent provirus. It is tempting to assign our observations to the failure of the immune system to eliminate the infected cell given the absence of proper expression of immunogenic proteins, in this case Tax. Tax is the major target of CTLs in HTLV-associated disease [25], and we found significant levels of Tax-specific CTLs in BLV-infected sheep (Van den Broeke, unpublished results). The lack of immunogenicity...
of naturally occurring tumors is often understood in terms of a suboptimal condition in the tumor microenviron-
ment to generate protective immunity, regulatory T-cell activity, dendritic cell dysfunction, production of suppressive factors such as IL-10, or changes in the pattern of antigen expression [1,3,26], but so far there was no example of complete suppression of tumor antigen expression, especially if this antigen is the major transforming protein.

The demonstration in S2531 of a link between the interrup-
tion of the long clinical latency and the complete sup-
pression of viral expression suggests that silencing is a late event in the multi-step process leading to the uncon-
trolled growth of a transformed B-cell clone and the onset of the fatal acute stage of the disease. Early after infection, cells that do not express viral proteins might have a sur-
vival advantage because they escape CTLs, but such cells will not outgrow the cells that express virus because of the absence of functional Tax protein capable of transactivat-
ing the host cell pathways responsible for enhanced B-cell proliferation. However, if virus silencing occurs when the cell has undergone sufficient events to reach a point of no return, impairment of immune surveillance might allow the uncontrolled proliferation of this fully-transformed B-cell clone. Whatever the mechanism – genetic or epige-
netic – it is critical for achieving complete silencing of all viral genes. Cellular changes that have occurred during the process of leukemogenesis are such that even the Tax oncoprotein can be turned off without reversing the trans-
formed phenotype. Loss of Tax and virus expression has been extensively documented in HTLV-1-associated dis-
ease and both genetic and epigenetic silencing mecha-

nisms have been described [13,27,28]. This study in sheep contributes to the further understanding of tumor-associa-
ted silencing. In particular, the analysis of sequential samples of the same individual from pre-tumoral to overt leukemia and the documentation of the timing of the Tax expression reduction are unique. Our findings are in strong contrast with observations in other viral-associated malignancies including HPV-, EBV-, and HBV-associated cancers, as well as tumors mediated by simple oncornavi-
ruses that all require sustained oncogene or transforming gene expression. This observation also raises a major con-
cern for the application of effective anti-tumor immuno-
otherapy. CTLs to the oncogenic protein might be effective when elicited during the chronic pre-leukemic stage, but would be irrelevant for eliminating malignant cells that do not longer express the initially-immunogenic target antigen after tumor progression.

Methods

Animals and animal samples
All sheep were housed at the Centre de Recherches Vétéri-
naires et Agrochimiques (Brussels, Belgium). Experimen-
tal procedures were approved by the Comité d'Ethique Médicale de la Faculté de Médecine ULB and were con-
ducted in accordance with national and institutional guidelines for animal care and use. S2531 was inoculated intradermally with 10⁷ PBMCs isolated from a BLV-
infected animal (S19) described earlier [8]. S267 was injected with naked proviral DNA of an infectious BLV variant (pBLVX3C) [9], isogenic to the full-length wild-
type 344 provirus used for in vivo infection of sheep [9,20-
23]. Blood was collected in EDTA-containing tubes and PBMCs were isolated using standard Ficol-Hyphaque sep-
neration. S267 lymphoid tumors were collected at necropsy, minced through a nylon mesh cell strainer (Becton-Dickinson) to obtain single-cell suspensions. Sheep used for injection with S267-derived cell populations were inoculated with 2 × 10⁷ BL267, L267, or CL267 respectively. Anti-p24 antibody titers and viral load were determined as previously described [8].

Cell cultures
PBMCs and single cell suspensions isolated from BLV-
infected sheep were cultured at a concentration of 10⁶ cells/ml in OPTMEM medium (Invitrogen) supple-
mented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids and 100 μg/ml kan-
amycin as previously described [8].

Southern blot, PCR, RT-PCR and sequence analysis
Genomic DNA was prepared and analyzed by Southern blot and PCR analysis as previously described [8]. The nylon-bound Sac I or EcoRI-digested genomic DNAs were hybridized with a 32P-labeled BLV full-length proviral DNA probe (Fig. 2A). Primers for PCR were as follow (nucleotide positions according to Sagata [29]: Tax1 [7321–7340]: 5’-GATGCCCTGGTCCGCCCTCTGT-3’, Tax2 [7604–7623]: 5’-ACCGTCGCTAGAGGCCGAGG-3’, U3 [8599–8618]: 5’-GCCAGACGCCCTTGGAGCGC-3’. Tax1-
Tax2 and Tax1-U3 were paired together for proviral DNA detection and sequencing respectively. For RT-PCR exper-
iments, total RNA was extracted using the Tripure reagent according to the manufacturer’s protocol (Roche). 1 μg of RNA was reverse transcribed and amplified using the Titan RT-PCR system according to the protocol supplied by the manufacturer (Roche). Primers EnvA [4766–4787]: 5’-TCCTGGCTACTAACCCCCCCGT-3’, and Tax2 were used for the detection of the 2.1 kb doubly-spliced tax/rex mRNA as previously described [8], generating a fragment of 482 bp (Fig. 2A). For provirus sequencing, amplifica-
tion of selected regions was performed using the Pfu proofreading DNA polymerase (Stratagene) and the puri-
fied products were sequenced using the Thermostequence radiolabeled terminator cycle sequencing method (GE Healthcare Biosciences).
Constructs and luciferase assays
DNA extracted from PBMCs isolated from S2531 at different times post-infection was amplified using primers Tax1/U3. Eco RI-restricted products were inserted into pSGTax [30] for exchange with the wild-type sequence. Each pSGTax2531 construct was used in HeLa co-transfection with pLTR-Luc, and luciferase activities were measured as described [19]. pSGTax contains the wild-type tax downstream of the CMV promoter; pLTR-Luc expresses the firefly luciferase under the control of the BLV-LTR promoter.

Proviral DNA from S2531 leukemic cells was cloned by insertion of EcoRI-restricted genomic DNA into the Lambda Dash® II vector (Stratagene) according to the manufacturer and used to evaluate the infectious potential in sheep.

Abbreviations
ATL: Adult T-cell Leukemia; B-CLL: B-cell Chronic Lymphocytic Leukemia; BLV: Bovine Leukemia Virus; EBV: Epstein-Barr Virus; HBV: Hepatitis-B Virus; HPV: Human Papilloma Virus; HTLV-I: Human T-lymphotropic Virus-1; MoMuLV: Moloney Murine Leukemia Virus; PRMCs: Peripheral Blood Mononuclear Cells; STLV: Simian T-lymphotropic Virus; WBC: White Blood Cell.

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
MM and PK set up the experiments, carried out most of the experimental work, and participated to the writing of the manuscript, MS participated in the transfection and luciferase assays, VC performed the cloning and sequencing experiments, PK was responsible for the interpretation of the results and corrected the manuscript, AVDB was the principal designer of the study, coordinated its realization and the writing of the manuscript. All authors read and approved the final manuscript.

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