Prostaglandin E₂ Increases Bovine Leukemia Virus tax and pol mRNA Levels via Cyclooxygenase 2: Regulation by Interleukin-2, Interleukin-10, and Bovine Leukemia Virus

DOHUN PYEON,† FRANCISCO J. DIAZ,2 AND GARY A. SPLITTER1*

Department of Animal Health and Biomedical Sciences1 and Endocrinology-Reproductive Physiology Program,2 University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 29 July 1999/Accepted 29 March 2000

Bovine leukemia virus (BLV), closely related to human T-cell leukemia virus type 1 (HTLV-1), is a type C retrovirus that infects bovine B cells and leads to enzootic bovine leukosis (16). The genomes of BLV and HTLV-1 are similarly arranged. In particular, the 3' region that contains the tax, rex, R3, and G4 genes is unique to BLV and HTLV-1 (2). In addition, there are several features of pathogenesis that are shared by BLV and HTLV-1. For both viruses, many infected individuals develop antibodies, but clinical symptoms are relatively rare. Disease progression in BLV-infected animals is divided into three stages: serologically positive, persistent lymphocytosis negative (alymphocytotic [AL]); and tumor-bearing stages (usually lymphocytotic [PL]). Most infected animals never display outward signs of disease and are referred to as asymptomatic or aleukemic. Fewer than 5% of infected animals develop malignant lymphosarcoma (11), while 30% of infected animals progress to persistent lymphocytosis, in which neoplastic B cells proliferate and leukocyte counts may exceed 10,000 cells/mm³ (17). Usually there is a long duration between these disease stages. The mechanism of disease progression from AL to PL or tumor-bearing stage is not clear.

Recent investigation has revealed that cytokine production plays a critical role in the progression of many different diseases (9, 32). In previous studies, we found cytokine polariza-

*Corresponding author. Mailing address: Department of Animal Health and Biomedical Sciences, University of Wisconsin-Madison, 1656 Linden Dr., Madison, WI 53706. Phone: (608) 262-1837. Fax: (608) 262-7420. E-mail: splitter@ahabs.wisc.edu.
†Present address: Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02115.
folicular dendritic cells. The functions of COX-2 and prostaglandins are very important in regulating normal physiological processes (8, 19), as well as the immune response. Here, we demonstrate that IL-10 decreased detection of COX-2 mRNA by PBMCs, while conversely, IL-2 increased COX-2 mRNA. Although PGE₂ reduced antigen-specific PBMC proliferation, PGE₂ increased detection of BLV and reversed the IL-10 inhibition of BLV tax and pol mRNA levels. In addition, BLV acts as an autocrine stimulator to increase the levels of BLV tax and pol mRNA and COX-2 mRNA.

COX-2 mRNA from PBMCs was inhibited by IL-10 and enhanced by IL-2. In a previous study, we found that human recombinant IL-10 (hrIL-10) inhibited BLV tax and pol mRNA, while IL-2 enhanced the detection of BLV tax and pol mRNA and BLV p24 protein in PBMCs in vitro (27). Also, IL-10-mediated regulation of BLV expression was macrophage dependent. Because COX-2, an important enzyme for prostaglandin synthesis, is mainly expressed by macrophages (18, 26, 36), we hypothesized that COX-2 and its products may affect BLV expression regulated by IL-2 and IL-10. Quantitative competitive PCR (QC-PCR) was performed to determine COX-2 mRNA levels in PBMCs cultured with or without hrIL-10. Transcripts of COX-2 mRNA were quantified by a competitive reverse transcriptase PCR (RT-PCR) assay using standard curve methodology. Validation of this assay and synthesis of native and competitor standards has been published previously (37, 38). A standard curve was created by RT-PCR using a constant amount of competitor RNA (2 aM) together with twofold serial dilutions of native RNA (15 to 0.11 aM). Unknown mRNA samples were diluted as needed, reverse transcribed and amplified with the same amount of competitor RNA, and compared to the standard curve. Reverse transcription was carried out in 1× RT buffer (Promega), 0.2 mM deoxynucleoside triphosphates, 100 μM random hexamer, and 40 U of Moloney murine leukemia virus RT for 1.5 h at 37°C, followed by 95°C for 10 min in a final volume of 20 μl. Four microliters of RT reaction mixture were then PCR amplified (30 s, 95°C; 30 s, 57°C; 30 s, 72°C) for 30 cycles, followed by 72°C for 5 min in 20 μl of 1× PCR buffer (Promega, Madison, Wis.), 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates, 0.4 μM upstream and downstream primers, and 0.5 U of Taq DNA polymerase. PCR products were separated on a 5% polyacrylamide gel electrophoresis (PAGE) gel and stained with ethidium bromide. Bands observed in gels were quantified using Collage software (Fotodyne, Heartland, Wis.). Densitometric values for the standard curve were plotted as follows: log[native RNA]/[competitor RNA] versus log[native RNA (aM)]. Addition of IL-10 reduced the detection of COX-2 mRNA in PBMCs from both AL and PL animals (Fig. 1a). These results paralleled the effects of IL-10 inhibition of BLV tax and pol mRNA levels (27). In contrast, IL-2 dramatically increased detection of COX-2 mRNA (Fig. 1b). These results suggest that cytokines, such as IL-2 and IL-10, can have opposing effects on the regulation of COX-2 mRNA.

PGE₂, a COX-2 product, enhances detection of BLV tax and pol mRNA and reverses IL-10 inhibition of BLV tax and pol mRNA. PGE₂ is the only COX-2 product expressed by macrophages (14). To determine whether PGE₂ affects BLV expression, BLV-infected PBMCs were cultured with or without PGE₂ in the presence of hrIL-10. Subsequently, the tax and pol mRNA produced by PBMCs from BLV-infected animals was measured by QC-PCR (27). For standardization, PCR was performed using this serially twofold diluted standard plasmid, with concentrations ranging from 8,192 to 16 fg/μl for tax and from 2,048 to 4 fg/μl for pol and with a fixed amount of mimic (10 fg/μl). Synthesized cDNA from each sample and fixed amounts of mimic were added to the same tube and amplified simultaneously with tubes for a standard reaction. Gel photographs were scanned, and the amplified DNA bands were analyzed by densitometry using the NIH Image program, version 1.61, with standard curves constructed with Cricket graph. The amount of cytokine produced was determined by comparing the density ratios of sample and standard reaction mixtures. As expected, we observed that different animals have different viral loads. Usually, PL animals have more viral load than AL animals. While IL-10 reduced detection of BLV tax and pol mRNA, PGE₂ reversed this IL-10 inhibitory effect (Fig. 2a). Addition of PGE₂ alone also enhanced BLV tax and pol mRNA levels (Fig. 2b). These results suggest that macrophage-derived PGE₂ may stimulate BLV expression from infected B cells and that IL-10 may inhibit BLV expression by reducing PGE₂ production. At the higher concentration of PGE₂, BLV tax and pol mRNA levels were slightly diminished, indicating that the stimulatory effect of PGE₂ was maximal at
1 μM. To confirm the effects of PGE₂ to increase BLV mRNA levels, a selective COX-2 inhibitor, NS-398 (21), was added to PBMC cultures. As expected, the level of BLV tax mRNA was dramatically suppressed by the COX-2 inhibitor (Fig. 2c). In contrast, addition of PGE₂ increased BLV tax mRNA detection regardless of NS-398 addition (Fig. 2c). These data suggest that a selective COX-2 inhibitor may reduce the level of BLV mRNA in infected B cells and that PGE₂ addition may bypass the NS-398 inhibition of COX-2 activity.

**Purified BLV increases the level of COX-2 mRNA and has an autocrine effect that increases the levels of BLV tax and pol mRNA.** Numerous factors can stimulate COX-2 and PGE₂ expression by macrophages. To investigate how BLV infection might affect COX-2 and BLV expression, BLV proteins were purified from the supernatant of the BL3⁺ cell line using metrizamide density gradient centrifugation. Purified BLV antigens were confirmed by sodium dodecyl sulfate-PAGE and immunoblotting with anti-gp51 and -p24 antibodies (data not shown). When purified BLV was added to PBMC cultures, COX-2 mRNA detection rapidly increased (Fig. 3a). Also, detection of BLV tax and pol mRNA increased by addition of purified BLV (Fig. 3b), suggesting that BLV antigens regulate BLV expression by an autocrine mechanism. To remove the possibility that other factors isolated during BLV antigen purification could affect COX-2 and BLV expression, a BLV-negative reagent was prepared from the BL3 cell line, which does not produce any BLV antigens. BLV gp51 and p24 were not detected by immunoblotting in the purified BLV-negative material. The BL3 purified material could not enhance COX-2 mRNA or BLV tax and pol mRNA (Fig. 3).

**PGE₂ suppresses antigen-specific PBMC proliferation.** To determine if PBMC proliferation and BLV expression were correlated, different concentrations of PGE₂ were added to the PBMC cultures with or without BLV tax mRNA and pol mRNA (Fig. 3).

**FIG. 2. PGE₂ increases detection of BLV tax and pol mRNA.** BLV-infected AL and PL PBMCs were cultured for 3 days with IL-10 (10 ng/ml) and increasing concentrations of PGE₂ (0.1 and 1 μM) (a). BLV-infected PBMCs were also incubated with PGE₂ (1 and 10 μM) alone (b) and/or NS-398 (20 μM) (c). QTC-PCR was performed as described in the text. The bands were analyzed using densitometry in the NIH Image program, version 1.61, and representative data are from one of three (a and b) or two (c) different AL and PL animals. Standard errors of means are shown from at least three experiments on cells from the same animal.
the absence of antigens was also slightly reduced by addition of PGE\(_2\) (Fig. 4). Thus, PGE\(_2\) enhanced BLV detection but suppressed antigen-specific PBMC proliferation. The concentration of cells differed between transcription and proliferation assays, as fewer cells were optimal in the U-bottom wells for the proliferation assay.

The results presented here demonstrate that IL-10 inhibited detection of BLV \textit{tax} and \textit{pol} mRNA and reduced COX-2 transcription from macrophages, while PGE\(_2\) activated BLV \textit{tax} and \textit{pol} mRNA. These data indicate that regulation of BLV is closely related to signals induced by PGE\(_2\). Both AL and PL animals, but not noninfected animals, produced similar findings when tested with IL-2, IL-10, and PGE\(_2\), supporting a common mechanism of pathogenesis in infected animals. Also, in spite of the relatively low IL-10 levels, AL animals produced more IL-10 than uninfected animals, and AL and PL animals responded similarly to IL-10 and PGE\(_2\). These findings suggest that macrophages produce PGE\(_2\) and have a central role in regulating BLV expression in infected B cells (Fig. 5).

The E series of prostaglandins are widely known as immunosuppressive products produced by macrophages, follicular dendritic cells, and fibroblasts (10, 17). These prostaglandins, especially PGE\(_2\), can downregulate many aspects of B- and T-cell functions. PGE\(_2\) production is triggered by inflammatory cytokines, such as IL-1 and TNF-\(\alpha\), that are produced in viral and bacterial infections (4, 20). Increasing PGE\(_2\) negatively regulates type 1 cytokines, such as IL-2, IFN-\(\gamma\), and TNF-\(\alpha\), by increasing production of type 2 cytokines, such as IL-10 (13). Thus, PGE\(_2\) may have a central role in regulating production of type 1 and type 2 cytokines. PGE\(_2\) activates a humoral immune response, stimulating B-cell differentiation and immunoglobulin class switching (26). In this paper, we show that PGE\(_2\) stimulated detection of BLV \textit{tax} and \textit{pol} mRNA and inhibited PBMC proliferation. These findings suggest that enhancement of BLV expression by PGE\(_2\) may not depend on cell proliferation. Ironically, B cells are the only PBMCs that are significantly infected with BLV (22), while macrophages are the only source of PGE\(_2\) in PBMCs (14). However, B cells...
have a number of PGE2 receptors that regulate B-cell activation (7). Signal transduction by PGE2 receptors mediates increased cyclic AMP (cAMP) production (3). BLV long terminal repeats (LTRs) contain a cAMP-response element (CRE) that facilitates BLV gene transcription (1, 39). Tax stabilizes CRE-binding protein (CREB) to bind CRE in LTRs (5). Thus, CREB and Tax may activate BLV expression of infected B cells. In addition, protein kinase C (PKC) increases BLV expression with increased Ca2+ influx (15). Therefore, increased PGE2 production by macrophages may stimulate BLV tax and pol mRNA expression through cAMP-dependent PKA and/or PKC signal transduction pathway. BLV LTRs also contain NF-κB binding sites that facilitate BLV transcription (6). Recently, antiinflammatory agents, such as aspirin and salicylate, reportedly inhibit the activity of IκB kinase-β, which facilitates the degradation of IκB and activates NF-κB (40). Antiinflammatory agents that inhibit prostaglandin synthesis may suppress BLV expression via NF-κB inhibition.

We demonstrate that BLV functions as a stimulant of COX-2 expression. Although PGE2 enhances IL-10 expression (13) to inhibit COX-2 and BLV expression, a synergistic effect of BLV expression, opportunistic infections (35), pregnancy (19), and/or stress (8) could induce disease progression in BLV infection. COX-2 and PGE2 also inhibit programmed cell death and facilitate tumor formation (33, 34), and thus these activities might promote lymphosarcoma and leukemia with other carcinogenic factors, such as Bcl-2 and BLV Tax protein, in BLV infection. Thus, in other retrovirus infections, the inhibitory function of IL-10 on human immunodeficiency virus expression has been reported (24, 30, 31). Most studies have utilized macrophage cell lines and primary macrophages, while the studies with T-cell lines or primary T cells failed to demonstrate the IL-10 inhibition of human immunodeficiency virus expression. Therefore, macrophages may have a direct role in regulating retrovirus expression responding to IL-10 (32). Our preliminary data showed that bovine herpes virus type 1 and Brucella abortus, two common opportunistic infections in cattle, activated COX-2 mRNA expression (D. Pyeon and G. A. Splitter, unpublished data). We anticipate that further research regarding PGE2 and opportunistic infections will reveal additional clues to solve the complicated mechanisms of disease progression in retrovirus infections.

To examine whether inhibitors of PGE2 or COX-2 would be efficacious for treatment of BLV infection, in vivo studies are necessary. Infected animals could be treated with a PGE2 inhibitor, such as indomethacin, and viral load and BLV expression could be measured. Treatment with a PGE2 inhibitor may reduce BLV load in BLV-infected animals and would support a role for PGE2 to stimulate BLV replication and disease progression in vivo. This study provides an additional strategy to treat retrovirus infection combined with currently available antiretroviral treatment.

This work was supported by National Cancer Institute grant R01 CA59127, BARD 95-34339-2556, and the College of Agricultural and Life Sciences.

REFERENCES


