

**CAPRINE ARTHRITIS ENCEPHALITIS VIRUS (CAEV)  
EXPRESSING THE SIV VPR/VPX GENES  
INDUCES PROPERTIES SIMILAR TO ANTI-CANCER DRUGS**

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The ability of the SIV mac239 *vpr* and *vpx* accessory genes to induce G2 phase arrest and apoptosis in the oncogenic process was tested. Primate lentivirus (HIV and SIV) *vpr* accessory genes encode 12-14 kDa proteins which induce specific cell cycle arrest at the G2 phase of infected cells preventing them from going through mitosis. Vpx is the second closely related protein encoded by the members of the HIV-2/SIVmac /SIVsmm group. Vpx and Vpr are critical for virus replication in non-dividing cells by participating in nuclear import of the pre-integration complex. Genomes of small ruminant lentiviruses: Caprine Arthritis Encephalitis Virus (CAEV) and Maedi Visna Virus (MVV), that are natural lentiviruses of domestic goat and sheep respectively, do not carry *vpr* and *vpx* genes. Thus, we developed a lentiviral chimeric virus based on the complete genome of CAEV in which the SIV mac239 *vpr* and *vpx* coding sequences were inserted. The resulting recombinant named CAEV-pBSCA<sub>vpxvpr</sub> was found to be infectious and replication competent. SIV Vpr/Vpx proteins were correctly and efficiently expressed in *in vitro* infected goat cells. Functional studies showed that SIV Vpr/Vpx proteins induce both specific G2 arrest of the cell cycle, and apoptosis in goat infected cells. Interestingly, following a high multiplicity of infection, both G2 arrest and apoptosis were found to be induced by Vpr/Vpx as early as 6 h post infection suggesting that the amount of SIV Vpr and Vpx proteins imported in the particles during the infection was sufficient to ensure such as functions. Data presented here highlight the evidence that proteins encoded by the accessory genes *vpr* and *vpx* govern two important functions (G2 arrest and apoptosis) which could be exploited as an appealing anti-cancer therapeutic approach that can be delivered with replication-defective CAEV vector.

Key words: Vpr/vpx, CAEV, SIV, cell cycle, cancer, therapy.

Cancer can be defined as a genetic disorder that has its origin from the accumulation of genetic mutations in the genome of target cells resulting in an uncontrolled cell proliferation and an abnormal homeostasis regulation.

Studies of human malignant disease showed that viruses are responsible for about 15% of human cancers and include the DNA tumour virus families

(papovaviruses, adenoviruses, herpes viruses, hepadna viruses) and the RNA oncogenic retroviruses (HTLV-1).

The accurate mechanisms and the cascade of events and the mediators implicated in oncogenesis still remain one of the main investigation areas of science. Nevertheless, two genetic events could be selected in this process. The first one includes all events that induce mutations in genes involved in the G1 checkpoint such as the loss of genes encoding for tumour suppressor products including *p53*, *Rb*, *P16<sup>INK4</sup>*, or over-expression of relevant oncogenes including MDM-2 and Cyclin D (7, 8, 18, 19, 21, 33). Only in exceptional conditions, some cancer cells possess the distributed G2 checkpoint instead G1 checkpoint (20). Thus, tumour formation occurs via the dysregulation of this regulatory process, in which tumour cells continue to divide out of control. The second class of genetic events includes those that confer a particular growth advantage to a target cell. Many data are supporting the concept of escape from apoptotic signals as a strategy often used in tumour progression process. Indeed, in a mouse model it has been demonstrated that tumour progression was associated with reduced susceptibility to apoptotic stimuli (27). On the other hand, the response to chemotherapy or radiation therapy in some cancers correlated with the induction of apoptosis within tumour cells (16).

While several therapeutic approaches have been developed to overcome such events, human cancers remain one of the major complex health problem over the world. The emergence of drug-resistance, and toxicity in some treated-patients, however, will remain a difficulty to overcome with the continued use of the currently available anti-oncogene agents. Therefore, alternative strategies to block the development of tumours either in place of or in combination with actual drugs are warranted. Toward this goal, we were interested in developing an alternative model as anti-cancer agent, based on gene therapy approaches, and that target cell cycle checkpoint and apoptosis processes.

One of the genes that could be a selected candidate is the primate lentivirus *vpr* accessory gene. *Vpr* is a unique gene to primate lentiviruses HIV and SIV. This gene encodes a 14 KDa viral protein R (Vpr), which is expressed in infected cells and packaged into assembled virions (9, 48). Vpr is required for the import of the viral protein-DNA integration complex into from the cytoplasm to the nucleus of non-dividing cells (15), and induces cell cycle arrest at the G2 phase in a variety of mammalian cells including transformed human cells (17, 42). Recently, it has been reported that Vpr is a strong apoptosis inducer in a variety of cell types including fibroblasts transformed tumour cells (43), T lymphocytes (47), monocytes and neurons (32)

SIV and HIV-2 but not HIV-1 genomes encode another accessory gene called *vpx* which has been shown to be crucial for productive infection of non-dividing cells *via* nuclear transport of pre-integration complex (12,; 31).

Since lentiviruses carrying therapeutic genes have one of the most important and required property to integrate their genome into that of the host cell, therefore to ensure stable long-term expression, in the present study we developed a lentiviral recombinant virus based on the infectious genome of the molecularly cloned CAEV and in which the SIVmac 239 *vpr* and *vpx* genes were inserted. Viruses derived from this recombinant genome were used to test the expression of SIV *vpr/vpx* genes and their interactions with goat cell proteins. Although, CAEV naturally infects goats it is a lentivirus closely related to the primate HIV and SIV. In contrast to HIV and SIV,

CAEV does not induce immunodeficiency in infected animals (5, 13). Indeed, the CAEV genome does not carry *vpr* and *vpx* accessory genes.

Our experiments showed that resulting recombinant virus named CAEV-pBSCAvpxvpr was shown to be infectious and replication competent and to produce virus titers equivalent to those obtained with the parental CAEV. SIV Vpr/Vpx proteins were correctly and efficiently expressed in *in vitro* infected goat cells.

Functional studies showed that SIV Vpr/Vpx proteins do induce G2 arrest of the cell cycle of infected goat cells. In addition, expression of SIV Vpr/Vpx proteins induces apoptosis of goat synovial membrane (GSM) cells. Interestingly, following a high multiplicity of infection, both G2 arrest and apoptosis induced by Vpr/Vpx were observed in concomitance as early as 6 hours post-infection, suggesting that the amount of SIV Vpr and Vpx proteins imported in the particles during the infection is sufficient to ensure such as functions. Results presented here demonstrated the ubiquitous conservation of SIV Vpr/Vpx protein functions from primate to small ruminant. This ubiquitous property is very important to develop strategies for multi-organs and multi-species strategies of therapy.

## Material and Methods

**Construction of a lentiviral recombinant genome capable of expressing SIV Vpr/Vpx.** The genome of the infectious molecular clone CAEV-pBSCA (26) was used to generate a recombinant genome in which the SIVmac239 *vpr* and *vpx* coding sequences were inserted. Firstly, *vpx* and *vpr* genes were amplified by PCR from the SIVmac239 genome, using the following primers: 5'-GCT GGC CAT CCT AGA CAG ACA AAA CTG GCA ATG GTA GCA ACA -3' and 5'-GCA GCG GCC CTG AAA GTA GTA AGC GAT GTC AGA TCC-3'. The 734 bp PCR product was sub-cloned into the pGEM-t plasmid DNA (Promega) to generate pGEM-vpxvpr construct. Then, a 734 bp fragment containing *vpx* and *vpr* coding sequences was isolated from the pGEM-vpxvpr following double digestion with *Eag*I and *Mse*I restriction enzymes. This fragment was inserted into the unique *Sma*-I site located at the end of *tat* and upstream of *env* coding sequences of the CAEV-pBSCA genome. The resulting recombinant plasmid DNA that contained *vpx* and *vpr* genes in the orientation of CAEV transcription was named CAEV-pBSCAvpxvpr.

**Determination of viral titers and infectivity.** Goat synovial membrane cells (GSM), originally obtained from explanted carpal synovial membrane of a colostrum-deprived newborn goat (39), were used to produce virus stocks, and as the source of indicator cells for quantification of both the infectivity and the cytopathicity of viruses used in this study. GSM cells were also used to test the ability of SIV Vpr/Vpx to induce G2 arrest and apoptosis.

Culture medium was collected at day 3, 5 and 7 post-transfection of GSM cells with the respective DNA using the lipofectamine method (39). Virus suspensions were tested for virus infectivity by correlative examination of induced cytopathic effects (giant multinucleated cells) in the indicator cells. Titrated viruses were fractionated in aliquots and stored at -80°C. Determination of titers of CAEV-pBSCA and CAEV-pBSCAvpxvpr was performed as follows: Samples from CAEV-pBSCA and CAEV-pBSCAvprvpx were serially diluted, and dilutions were used to inoculate the indicator GSM cells. At 6 d. post-infection, the cell monolayers were stained with May-

Grünwald Giemsa, and examined for the presence of syncytial cytopathic effect (giant multinucleated cells). The titers were calculated using the Reed-Muench method, and expressed as the tissue culture infectious dose (TCID<sub>50</sub>) per milliliter of supernatant.

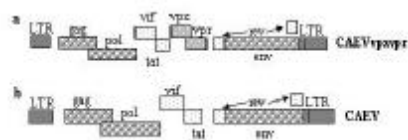
**Radioimmunoprecipitation assay.** Immunoprecipitation of virus-specific proteins was performed as previously described (4, 6). Briefly, GSM cells were seeded at day 0 into six-well plates at a density of  $1 \times 10^5$  cells/well. At day 1, the monolayers were inoculated with CAEV-pBSCA, and CAEV-pBSCAvprvpx at a multiplicity of infection (m.o.i) of 0.1 and incubated at 37°C in 5% CO<sub>2</sub>. At day 7, cell monolayers were rinsed twice with serum free MEM, and incubated for 2 h in methionine/cysteine free MEM and then radio-labeled overnight with 100 µCi/ml of (<sup>35</sup>S) methionine/cysteine (Promix, Amersham, Orsay, France) into 1 ml of the same medium for 18 h. CAEV-pBSCA specific proteins were immunoprecipitated using the hyperimmune serum (G9615) from a goat that had received several injections of mixture of three different CAEV and MVV K1514 isolates. SIV Vpr/Vpx proteins were immuno-precipitated with an immune rabbit polyclonal antibody directed against Vpx and Vpr proteins that we produced following immunization of a rabbit with 1 mg of gel purified Vpx and Vpr proteins. Immunoprecipitated proteins were then separated by Sodium-Dodecyl-Sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and virus proteins released into the culture medium, and or present in the cell lysate fractions were visualized by standard autoradiography.

**Detection of the effect of SIV Vpr/Vpx on the cell cycle arrest.** DNA cell cycle analysis was measured by propidium iodide (Sigma, ref: 4170)-stained nuclei by using FACScan as previously described (36). Briefly, infected GSM were harvested at different time post-infection, resuspended into 1 ml of 1X PBS, and fixed in 100% ethanol. After 30 min incubation in ice, cells were rinsed once with 1XPBS, incubated for 30 min at room temperature in a solution containing RNase A (10 mg/ml), and then stained by addition of 10 mg/ml of propidium iodide. The fluorescence of 10000 cells was analyzed by Facscan flow cytometry (Becton & Dickinson) using the lysis II software. Data are presented as the G2/G1 ratio.

**Apoptosis analysis.** GSM cells were infected with CAEV-pBSCAvpxvpr recombinant virus and the parental CAEV-pBSCA at a low and high m.o.i. (1 and 5). Viral apoptotic activity was determined using the Apostain Mab to ssDNA (F7-26) commercial kit (Alexis Biochemicals, Product No.804-192-L001). This method is designed to detect and quantify apoptosis at the single-cell level after DNA denaturation by heating at a low temperature in the presence of formamide and staining with a monoclonal antibody (Mab) F7-26 specific to single-stranded DNA (ssDNA). Both attached and floating cells were Apostain Mab stained at different time of infection and  $5.10^5$  cells were analyzed by flow cytometry. As a positive control, GSM were stimulated to undergo apoptosis by treatment with 20 µM of staurosporine (Sigma, ref.S4400). Non-infected GSM were used for each experiment as a negative control. The percentages of apoptotic cells reported in each figure were defined as the cells fluorescein labeled DNA breaks.

## Results

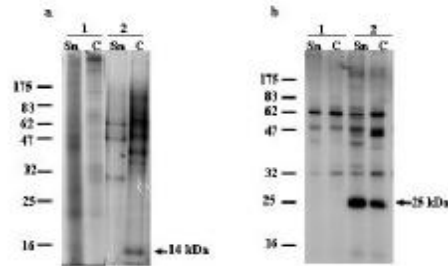
**CAEV-pBSCAvpxvpr is replication competent and can infect productively GSM cells.** The construction of CAEV-pBSCAvpxvpr recombinant virus (Fig. 1a) was based on the full length infectious molecularly cloned genome derived from the CO strain of CAEV, named CAEV-pBSCA (Fig. 1b) in which SIV *vpr* and *vpx* genes were inserted. To study the properties of CAEV-pBSCA recombinant virus carrying the SIV *vpr/vpx* genes and to evaluate its potential use as a therapeutic agent, the infectivity of the produced recombinant virus CAEV-pBSCAvpxvpr was tested *in vitro*. Using the Reed Muench method, we showed that virus stocks in the culture medium harvested at day 7 post transfection were found to contain titers equivalent to  $10^6$  and  $10^7$  TCID<sub>50</sub>/ml for CAEV-pBSCAvpxvpr recombinant virus and similar or slightly lower ( $10^5$ - $10^6$  TCID<sub>50</sub>/ml) for the parental CAEV-pBSCA. These results indicated that despite the addition of a 0.8 kb DNA segment (10% of the size of CAEV genome), CAEV-pBSCAvpxvpr was fully replication competent. It has been well documented that CAEV adapted laboratory strains efficiently replicate in GSM cells. Virus replication is associated with development of cytopathic effect (CPE) that is characterized by syncytium formation (giant multinucleated cells) (4, 28, 40, 46). Like CAEV-pBSCA, CAEV-pBSCAvpxvpr was found to be infectious and replication competent. However, infection with CAEV-pBSCAvpxvpr was associated with development of greater cytopathic effect induction compared to those observed in CAEV-pBSCA infected GSM.



**Fig. 1.** Genome organization and strategy of construction of CAEV-pBSCAvpxvpr

a) Schematic representation of the map of pBSCAvpxvpr recombinant virus. To produce CAEV-pBSCAvpxvpr recombinant virus, *vpx* and *vpr* genes were amplified by PCR from SIVmac 239. PCR product was cloned inside of the spacer sequences located between the *tat* and *env* genes of CAEV-pBSCA (b) a plasmid which carried the complete genome of the infectious CAEV.

**SIV Vpr/Vpx proteins are expressed in GSM infected cells.** SIVmac239 Vpr/Vpx and CAEV-pBSCA-specific proteins were immunoprecipitated from the supernatants and the cell lysates using the respective anti-Vpr/Vpx antiserum and anti-goat hyperimmune serum. As shown in Fig. 2a lane 2, a protein with an apparent molecular weight of 14 kDa was immunoprecipitated exclusively from the cell lysate of CAEV-pBSCAvpxvpr infected GSM cells and not from any supernatant. While no signal was detected when Vpx/Vpr antiserum was used to immunoprecipitate proteins from non-infected GSM cells (Fig. 2a, lane 1). In contrast to SIV proteins, CAEV-pBSCA specific radiolabeled proteins were immunoprecipitated from both supernatant and cell lysate of GSM cells infected with CAEV-pBSCA virus (Fig. 2b, lane 2), but not from non-infected GSM (Fig. 2b, lane 1). These results clearly indicate that SIV Vpr/Vpx proteins were correctly expressed in goat infected cells.



**Fig. 2.** Radio-immunoprecipitation of virus-specific proteins.

Radiolabeled proteins were immunoprecipitated from goat synovial membrane cells infected with CAEV-pBSCAvpxvpr as described in Material and Methods. At day 5 post-infection, proteins were radiolabelled overnight by addition of 100  $\mu$ Ci of ( $^{35}$ S-

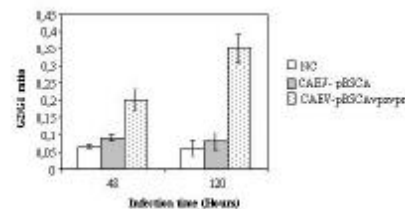
methionine and cysteine) in the culture medium of infected cells. Proteins from supernatant fluid and cell lysate were immunoprecipitated using specific antibodies. Proteins were separated using SDS-PAGE (12.5%) and then visualized using standard autoradiography technique. (a) Immunoprecipitation from supernatant (sn) and cell lysate (c) from non-infected (lane 1) and pBSCAvpxvpr infected cells (lane 2) immunoprecipitated with a polyclonal-rabbit anti-Vpx/Vpr serum. (b): Immunoprecipitation from supernatant (sn) and cell lysate (c) from non-infected (lane 1) and CAEV-pBSCA infected cells (lane 2) using hyperimmune serum (G9615). Molecular mass in kilodaltons of pre-stained markers is shown on the left. The 14 kDa Vpx/Vpr proteins and the 25 kDa CAEV gag protein are indicated to the right of each photograph.

### SIV Vpr/Vpx proteins induce G2 arrest in *in vitro* GSM infected cells.

Cancer has been viewed as a result of the loss of cell cycle regulators, thus cancer cells continue to divide in an uncontrolled manner. Since SIV Vpr has been found to be able to specifically block the cell cycle progression in primate system, we tested whether this function is conserved in caprine cells. GSM cells were inoculated at m.o.i of 1 with CAEV-pBSCAvpxvpr and analyzed for their DNA content by flow cytometry at 48 and 120 h after infection. Non-infected and CAEV-pBSCA infected GSM cells were used as controls.

**Fig. 3.** SIV Vpr/Vpx proteins induce G2 cell cycle arrest in GSM infected cells

Goat synovial membrane cells (GSM) were infected with CAEV-pBSCA and CAEV-pBSCAvprvpx at multiplicity of infection of 1. Non-infected cells were used as negative control (NC). Cells were harvested at 48h and 120 h after infection and stained for DNA content with propidium iodide. Cells were then analyzed by flow cytometer using a Facscan, over 10.000 events.

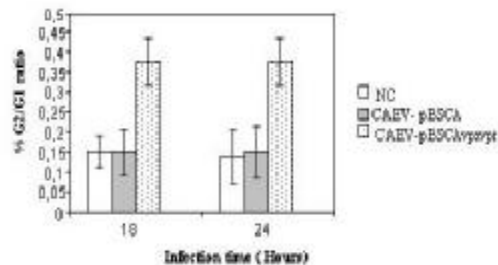


The ratios of the percentage of cells in G2 to percentage of cells in G1 phases, are reported. Data were produced by using lysis II analysis Software.

Examples of cell cycle profiles are shown in Fig. 3. For simplicity, the results are presented as the ratio of the percentage of cells in G2 relative to the percentage of cells in G1 of the cell cycle. We found that only GSM cells infected with CAEV-pBSCAvpxvpr showed a higher proportion of cells arrested in the G2 phase of their cell cycle, while non-infected and CAEV-pBSCA did not. At 48 h post-infection, the ratio of cells in G2 compared to cells in G1 were found to be 0.09 and 0.06 for cells

infected with CAEV-pBSCA and non-infected cells respectively, while, a higher ratio (0.2) was observed in cells infected with CAEV-pBSCAvpxvpr. At 120 hours post-infection a significant increase of the rate of cells arrested in the G2 phase was observed through the expression of SIV Vpr/Vpx proteins. Indeed, ratio equivalent to 0.06 and 0.08 for non-infected cells and cells infected with CAEV-pBSCA respectively were observed, while a ratio of 0.35 was observed with cells infected with pBSCAvpxvpr. This result is the first demonstration that SIV Vpr and Vpx proteins are able to induce a specific G2 arrest of cell cycle in cells other than those of primates and yeast.

As shown in Fig. 4, when GSM cells were inoculated at a high multiplicity of infection (m.o.i = 5), SIV Vpr/Vpx induced G2 cell cycle arrest at a very early stage of infection. At 18 h post inoculation a three fold increase of G2/G1 ratio was observed with GSM cells inoculated with CAEV-pBSCAvpxvpr compared to the ratios obtained with cells inoculated with CAEV-pBSCA, or non-infected cells. Similar data were observed at 24 h post-infection suggesting that the *de novo* synthesis and action of SIV Vpr and Vpx occurred later than 24 h. This result suggests that the amount of SIV Vpr and Vpx proteins imported in the particles during the infection of cells may be sufficient to induce a G2 cell cycle arrest in goat cells.



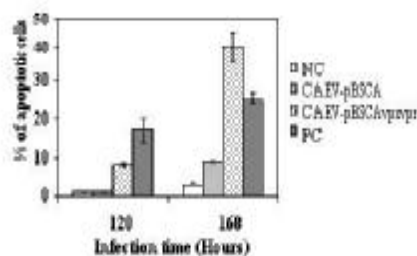
**Fig. 4.** SIV Vpr/Vpx proteins induce G2 cell cycle arrest in GSM infected cells at early time post-infection.

GSM cells were infected with CAEV-pBSCA and CAEV-pBSCAvpxvpr at a multiplicity of infection of 5. Non-infected cells were used as negative control (NC). 18 and 24 h post-

infection cells were stained with propidium iodide for DNA content analysis. DNA quantification was performed by flow cytometry analysis using Lysis II analysis Software. For simplicity, the data shown are indicated as the ratio of the percentage of cells in G2 to percentage of cells in G1.

**SIV Vpr/Vpx proteins are strong apoptosis inducers in GSM cells.** Several groups have reported a higher incidence of tumor development in human and in mice with genetic alteration of apoptotic mediators, such as the loss of Caspase-1 and 3, the over-expression of Bcl-2 or the deletion of Fas ligand (23, 30). Since SIV Vpr but not Vpx has been described as an apoptotic inducer protein, it is of interest to exploit this finding to use this protein as an anti-cancer agent. In this context we tested the capacity of both SIV Vpr and Vpx expressed in the CAEV recombinant virus to induce GSM cells apoptosis. GSM cells were infected with CAEV-pBSCAvpxvpr recombinant virus and the parental CAEV-pBSCA at m.o.i = 1. The rate of DNA breaks was assessed by flow cytometry following staining of cells with a monoclonal antibody specific to single-stranded DNA, that as detected with a secondary FITC conjugated monoclonal

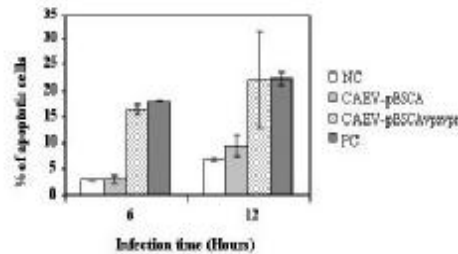
antibody. As shown in Fig. 5, approximately 8% of cells infected with CAEV-pBSCAvpxvpr underwent apoptosis at 120 H post-infection. Similarly, when cells had been treated with staurosporine the percentage of apoptotic cells was 17%. While, the percentage of apoptosis remained at a low level in non-infected and CAEV-pBSCA infected cells (1.12% and 1.16%, respectively). Interestingly, the rate of apoptosis cells continued to increase significantly until 168 h post-infection in CAEV-pBSCAvpxvpr (40%) and staurosporine inoculated cells (25%), and did not change significantly in non infected (3%) cells. An increase in the percentage of apoptotic cells was observed in GSM infected with the parental CAEVpBSCA (8%). Nevertheless, it remained significantly lower than that observed in infected cells expressing the SIV Vpr/Vpx proteins, indicating the strong capacity of SIV Vpr/Vpx to induce apoptosis.



**Fig. 5.** Analysis of SIV Vpr/Vpx proteins effect on apoptosis induction in GSM infected cells.

GSM cells were infected at an m.o.i. of 1 with CAEV-pBSCA and CAEV-pBSCAvpxvpr viruses. At 120 h and 168 h post-inoculation, the cells were stained with a monoclonal antibody specific to single-stranded DNA, and then with an FITC conjugated-monoclonal antibody. The rate of DNA breaks was assessed by flow cytometry and then analyzed by flow cytometry. Results are presented in graphics as the percentage of GSM that were FITC positive (Apostain).

To test whether SIV Vpr/Vpx can induce apoptosis early after infection, GSM cells were infected with CAEV-pBSCA and CAEV-pBSCAvpxvpr at a high multiplicity of infection (m.o.i = 5), and analyzed for DNA breaks at different time post-infection. The results of this study showed that apoptosis was induced within 6 h post-infection (Fig. 6). 16.33% of CAEV-pBSCAvpxvpr infected cells underwent in apoptosis, and this rate of apoptotic cell increase significantly (33%) 6 h later (Fig. 6). Similarly a high percentage of apoptotic cells was obtained when cells were treated with Staurosporine (Fig. 6, 18% and 22.23% at 6 h and 12 h, respectively). In contrast to these results, Apostain staining of non-infected and CAEV-pBSCA infected cells remained lower than that observed with CAEV-pBSCAvpxvpr throughout the time course of the experiment. These results showed that expression of SIV Vpr/Vpx led to apoptosis of goat cells at early and at the late stage of infection. At the early stage of infection, apoptosis is more likely induced by Vpr/Vpx proteins imported into the viral particles instead of the *de novo* synthesised viral proteins in the infected cells.



**Fig. 6.** SIV Vpr/Vpx proteins induce GSM cells apoptosis early after infection.

The rate of apoptotic GSM cells was determined 6 H and 12 H after infection with CAEV-pBSCA and the recombinant virus CAEV-pBSCAvpxvpr at a m.o.i. of 5. Cells were stained using the Apostain method as described in materials and methods. The rate of DNA breaks was assessed by flow cytometry. Results are presented in graphics as the percentage of cells that were FITC positive (Apostain).

### Discussion

The data here presented established the importance of the SIV *vpr/vpx* accessory genes as potential tools to be used as anti-cancer agents, since their expression is associated with the G2 cell cycle arrest and apoptosis of cells, which are two important activities that are currently used as target approaches to develop anti cancer strategies.

We constructed a lentiviral recombinant virus based on the genome of CAEV in which we inserted the SIV *vpr/vpx* coding sequences. The resulting recombinant virus named CAEV-pBSCAvpxvpr was tested for its ability to induce the specific G2 arrest of the cell cycle and regulation of apoptosis in caprine cells. We found that the recombinant virus was infectious and replication competent equivalently to the parental CAEV-pBSCA. SIV Vpr/Vpx proteins were also found to be correctly and efficiently expressed in infected goat cells. We reported that SIV Vpr/Vpx proteins are not only expressed in caprine cells, but also are functional, since they induced G2 cell cycle arrest and apoptosis.

Altered cell cycle control has emerged as a recurring area of interest in oncogenesis studies. Strategies that would repair the cell cycle control in tumour cells have appeal as a novel emerging concept in cancer therapy. Available anti-cancer therapies (chemotherapy, radio-ionization) kill proliferating cancer cells by damaging their DNA and inducing apoptosis. However, several limitations have been observed, since many tumour cells developed resistance to DNA-damage induced cell killing. Regarding cellular response to DNA damage, it involved cell cycle arrest mainly at G1 and G2 phases, allowing cells to repair their DNA before entering the S-phase for DNA replication and M-phase for mitosis and to maintain the cell integrity. Development of G1 arrest-triggering therapeutic drugs is limited by the fact that G1 arrest requires functional *p53* (45), and that more than 50% of human cancers have mutations in the tumour suppressor gene *p53* (21). Thus, G2 phase control abrogators are potentially an important alternative and a currently new class of anti-cancer drugs.

Towards such approaches we tested the ability of SIV Vpr/Vpx proteins to induce cell cycle G2 arrest in caprine cells in order to evaluate its anti-cancer potential as a G2 abrogator-mediating cancer therapy. Indeed, it has been clearly established that SIV and HIV-2 Vpr induces cell cycle arrest at the G2 phase not only in human and simian cells (1, 3, 11, 17, 34); but also in fission yeast *Schizosaccharomyces pombe*

over-expressing HIV-1 Vpr (49, 50). Vpr-associated G2 arrest of the cell cycle was found to be associated with the inhibition of activation of the p34cdc2-cyclin B complex by increasing its phosphorylation at specific sites both in fission yeast and human cells (14, 22, 35). In the present study we asked whether Vpr-inducing G2 arrest is conserved in mammalian non-primate cells. We found that SIV Vpr/Vpx proteins are able to induce specific G2 cell cycle arrest in caprine cells, suggesting that this function was not restricted to primate and yeast cells only. This finding increases the potential of use of Vpr as a new complementary drug to anti-cancer chemotherapy either in human and non-human cancer. It is of interest to note that cell-cycle mediating drug resistance is best described as a relative insensitivity to chemotherapeutic agents, a concept that is best exemplified when these agents are used in combination. For instance, Paclitaxel, which is an anti-cancer agent that induces G2-M arrest, is currently used in patients with ovarian breast, lung, head, neck, and oesophageal cancers (37) when combined with Flavopiridol they show emergence of drug-resistance in patients with gastric and breast cancer (25). This was shown to be resulting from antagonism activities allowing diminution of sensitivity of tumours cells to cell cycle regulation. Therefore, testing alternative strategies is strongly needed to overcome this type of limitation. The results described in the present study provide the evidence that SIV *vpr/vpx* genes-inducing G2 cell cycle arrest could be an appropriate candidate to develop new therapeutic approaches which can be combined with cytotoxic chemicals or radiation. This may greatly help to fight against some complex cancers, particularly those associated to *p53* gene mutation.

The pathobiology studies of tumours revealed its association not only with dysregulated proliferation state but also to inability of cells to undergo apoptotic death (41, 44). Development of several apoptosis therapeutic strategies is currently evaluated at the clinical level. Nevertheless, toxicity of such type of drugs is the major obstacle that limits the success of clinical development of apoptosis-triggering drugs. For instance, TNF and Fas that are strong apoptosis-inducers have been tested in rats in combination to chemotherapy and were shown to induce ischemic and haemorrhagic lesions in several tissues (10). Alternative, apoptosis-triggering agent approaches which can be potentially targeted to tumour cells, are then in urgent need. In our experiments we reported that SIV Vpr/Vpx induce a significant increase in the number of caprine apoptotic cells. This property brings additional argument that these proteins might have a potential use to target apoptosis to kill transformed cells in cancer therapy.

In our experiment SIV Vpr/Vpx are expressed in a CAEV recombinant virus that found to be replication competent in caprine cells. It is interesting that this virus, when are pseudotyped with the polytropic envelope of VSV, it acts like a single round virus since there no functional receptors in the surface of human cells for this virus (26). It is clear that improvements are needed if CAEV-based chimeric virus will be selected for delivery of Vpr/Vpx proteins, and then to test its anti-oncogene activity on a variety of cell types. One of the key elements, which we observed in this study, is that the very low amount of SIV Vpr/Vpx associated to viral particles was sufficient to induce both G2 arrest and apoptosis early after cell inoculation, demonstrating that SIV proteins could be functional even in the absence of their *de novo* expression, which will have a beneficent implications in developing of CAEV-pBSCA replication-defective vectors expressing *vpr/vpx* genes.

Cancer gene therapy approaches that directly target tumour cells are yet under investigations, and include cytokine gene transfer, inactivation of proto-oncogene expression, and restoration of defective tumour suppressor genes. The majority of these

approaches have been developed using adenoviral vectors. Results obtained with adenovirus vectors targeting regulators of cell cycle and or apoptosis (24, 38) are encouraging particularly in the treatment of neck (2) and lung cancer (29). However, retroviral vectors were less used in clinical trials to deliver anti-cancer genes; while they are likely to be most promising vehicles due not only to their ability to integrate the therapeutic gene into the host genome, but also they can induce a stable long term-expression of the therapeutic gene.

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