The HIV-1 Vpr displays strong anti-apoptotic activity

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Abstract Mutations in the human immunodeficiency virus type 1 (HIV-1) *vpr* gene only slightly reduce the replication rate of the virus. To study the role of HIV-1 Vpr in biological effects on cells, HEp-2 cells, which express HIV-1 Vpr constitutively but at a low level, were established. While control HEp-2 cells underwent apoptosis when incubated with sorbitol, the morphological and biochemical apoptotic changes were inefficiently induced in the HIV-1 Vpr-expressing cells by the same treatment. These results clearly indicate that HIV-1 Vpr has anti-apoptotic activity, and raise the possibility that Vpr acts as a weak activator of virus replication through anti-apoptosis.

Key words: Human immunodeficiency virus type 1; Vpr; Apoptosis; Anti-apoptosis

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) carries a number of unique genes in its genome not found in simple retroviruses [1–4]. These are two regulatory [2] and four accessory genes [3,4]. Of the four accessory proteins, Vif, Vpu, and Nef enhance viral infectivity at least in certain cell types [3,4], and have thus clear functional roles for viral replication. However, it is uncertain whether Vpr much affects the virus replication in various types of cells [5–9]. In contrast to its role in viral replication, Vpr has now been recognized as a key to crucial interactions between HIV-1 and host cells [3,4]. HIV-1 Vpr arrests cells in the G_2 phase of the cell cycle [10–13], induces cell differentiation [14], and is responsible for the regulation of apoptosis [15–18]. Contrasting results have been reported for the effect of HIV-1 Vpr on apoptosis and anti-apoptosis [15–18].

In this report, to investigate interactions between HIV-1 Vpr and cells, HIV-1 *vpr* was introduced into HEp-2 cells to obtain cells constitutively expressing Vpr. The HEp-2 cell system has been shown to be very sensitive for detection of apoptosis [19,20]. We analyzed here the ability of HIV-1 Vpr to regulate apoptosis by this HEp-2/Vpr system.

2. Materials and methods

2.1. Cells and transfection

A human epidermoid carcinoma cell line HEp-2 (ATCC CCL23) and an SV40-transformed African green monkey kidney cell line COS-7 (ATCC CRL1651) were maintained in Eagle's minimal essential medium supplemented with 10% fetal calf serum. For transfection, uncleaved plasmid DNA was introduced into cells by the calcium-phosphate coprecipitation method [21].

2.2. DNA constructs

The expression plasmid pME18Neo-Fvpr carrying the Flag and HIV-1 *vpr* sequences has been previously described [22]. A control plasmid pME18Neo lacking the HIV-1 *vpr* sequence was constructed by removing the *Xho*I-*Not*I fragment from pME18Neo-Fvpr.

2.3. Western blotting

Western immunoblotting was performed as previously described [23]. Vpr was detected by the ECL system (Amersham) using anti-Flag monoclonal antibody M2 (International Biotechnologies).

2.4. RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from cells with Isogen (Nippon Gene), and complementary DNA (cDNA) was synthesized from 1 μg of the RNA with the First-Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR was carried out using the TaKaRa Taq system (Takara Shuzo) essentially as previously described [17]. The primers for HIV-1 *vpr* (Vpr-1 and Vpr-2) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown in Table 1.

2.5. Assays for apoptosis

Apoptosis was induced in HEp-2 cells by treatment with sorbitol as previously described [19]. Apoptosis was monitored morphologically by cell shrinkage and biochemically by fragmentation of chromosomal DNA into nucleosomal oligomers as previously described [19,20].

3. Results

3.1. Establishment of HEp-2 cells expressing HIV-1 Vpr

To study the biological effects of HIV-1 Vpr on cells, particularly the ability of Vpr to regulate apoptosis, a Vpr expression vector designated pME18Neo-Fvpr was transfected into HEp-2 cells and COS-7 cells, which are very sensitive to transfection analysis. A plasmid designated pME18Neo served as negative control. Fig. 1A,B shows the transient expression of HIV-1 Vpr as monitored by Western blot analysis of HEp-2 and COS-7 cells transfected with the vectors. HIV-1 Vpr was readily detected in HEp-2 cells as well as in COS-7 cells. After confirmation of the transient expression of Vpr in HEp-2 cells, transfected cells were cultured in the presence of 300 μg of G418 per ml, and surviving cells were collected after 2 weeks. Cells were pooled to avoid clonal selection of cells with an extreme phenotype. Following G418 selection, lysates were prepared from HEp-R (transfected with pME18Neo-Fvpr) and HEp-C (transfected with pME18Neo) cells, and subjected to Western blot analysis. As shown in Fig. 1C, no specific Vpr expression was detected in HEp-R cells. The experiments were repeated with the same negative results.

A low level of HIV-1 Vpr expression in HEp-R cells might account for the results described above. We therefore monitored the expression of Vpr-RNA by RT-PCR using the primer pairs indicated in Table 1. As shown in Fig. 2, the RNA

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Table 1
Primers used for RT-PCR in this study

Gene	GenBank no.	Sequence of primers		Products (bp)
Vpr-1				
Sense	M19921	5′-GCCATACAATGAATGGACACTAG-3′	(5597–5619)	244
Antisense		5′-ACTGGCTCCATTTCTTGCTCTC-3′	(5819–5840)	
Vpr-2				
Sense	M19921	5′-GAAGACCAAGGGCCACAGAG-3′	(5574–5593)	264
Antisense		5′-GGCTCCATTTCTTGCTCTCCT-3′	(5817–5837)	
GAPDH				
Sense	M33197	5′-ACGCATTTGGTCGTATTGGG-3′	(86–105)	231
Antisense		5′-TGATTTTGGAGGGATCTCGC-3′	(297–316)	

of HIV-1 Vpr was easily detected in HEp-R but not in HEp-C cells. The negative results obtained without RT in this assay substantiated the expression of Vpr RNA in HEp-R cells.

In total, we concluded that a low level of HIV-1 Vpr is expressed in the HEp-R cells.

3.2. Anti-apoptotic nature of HEp-R cells

We examined the apoptotic responses of HEp-R and HEp-C cells to sorbitol treatment. We previously demonstrated that parental HEp-2 cells efficiently and rapidly undergo apoptosis when treated with sorbitol [19]. HEp-2 cells, therefore, can be used for detection of anti-apoptotic activity of viruses [19]. The apoptosis was monitored morphologically and biochemically as previously described for HEp-2 and HeLa cells [19,20,24]. As shown in Fig. 3, morphological changes of HEp-C cells such as rounding and shrinkage were easily observed shortly after sorbitol treatment as previously described for HEp-2 cells. In contrast, HEp-R cells were quite resistant to the sorbitol treatment, and a relatively minor portion of cells displayed morphological changes. These experiments were repeated with essentially the same results. To determine whether biochemical apoptotic changes were induced in cells, the degree of DNA fragmentation was quantitatively determined. As shown in Fig. 4A, HEp-C and HEp-R cells were clearly different with respect to the induction of nucleosomal DNA ladder by sorbitol. HEp-R cells were more resistant than HEp-C cells to the sorbitol treatment. In good agreement with this observation, after sorbitol treatment, a smaller proportion of fragmented DNA was found in HEp-R cells than in HEp-C cells (Fig. 4B).

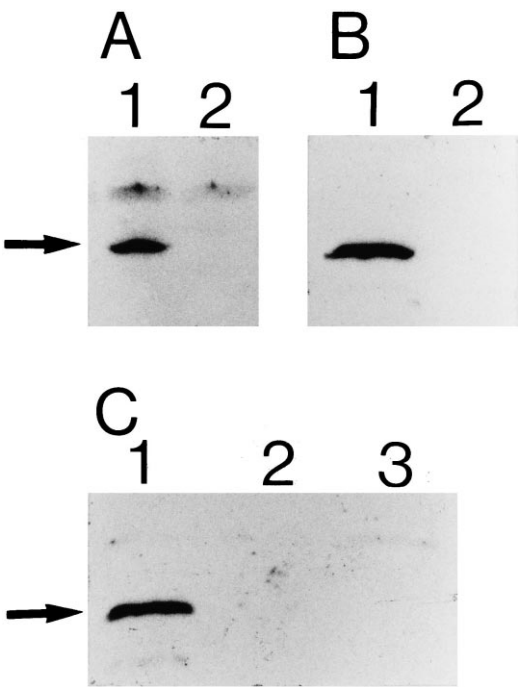

Fig. 1. Expression of *vpr* as monitored by Western blotting. HEp-2 (A) and COS-7 (B) cells were transfected with pME18Neo-Fvpr or pME18Neo, and 2 days later lysates were prepared for Western blot analysis as previously described [23]. Cell lysates prepared from HEp-R (stably transfected with pME18Neo-Fvpr) and HEp-C (stably transfected with pME18Neo) cells selected by G418 were also analyzed (C). Lanes in A and B: 1, Vpr expression vector pME18-Neo-Fvpr; 2, negative control pME18Neo. Lanes in C: 1, COS-7 cells transiently transfected with pME18Neo-Fvpr; 2, HEp-R cells; 3, HEp-C cells. Arrows indicate the position of the Flag-Vpr protein.

4. Discussion

In this report, we have demonstrated that HEp-2 cells expressing the HIV-1 Vpr protein (HEp-R) are relatively resistant to the induction of apoptosis by sorbitol. This observation supports the notion of Conti et al. that HIV-1 Vpr is a negative regulator of apoptosis [17]. They have demonstrated that a low level of Vpr expression detectable by Western blotting is sufficient to render cells resistant to apoptosis [17]. We showed

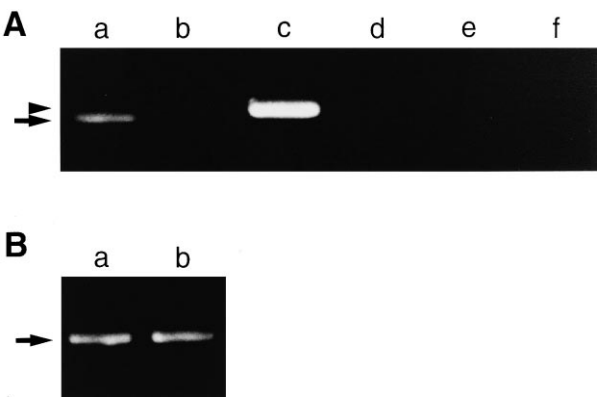

Fig. 2. Expression of *vpr* as monitored by RT-PCR. Total RNA was extracted from HEp-C and HEp-R cells and subjected to RT-PCR analysis using primer pairs listed in Table 1. Amplified products were run through a 3% agarose gel and visualized by ethidium bromide staining. Lanes in A: a, HEp-R RNA (Vpr-1); b, HEp-C RNA (Vpr-1); c, HEp-R RNA (Vpr-2); d, HEp-C RNA (Vpr-2); e; HEp-R RNA without RT (Vpr-1); f, HEp-R RNA without RT (Vpr-2). The arrowhead and arrow indicate the positions of 264 and 244 bp DNAs, respectively. Lanes in B: a, HEp-R RNA (internal control GAPDH); b, HEp-C RNA (internal control GAPDH). The arrow indicates the position of 231 bp DNA.

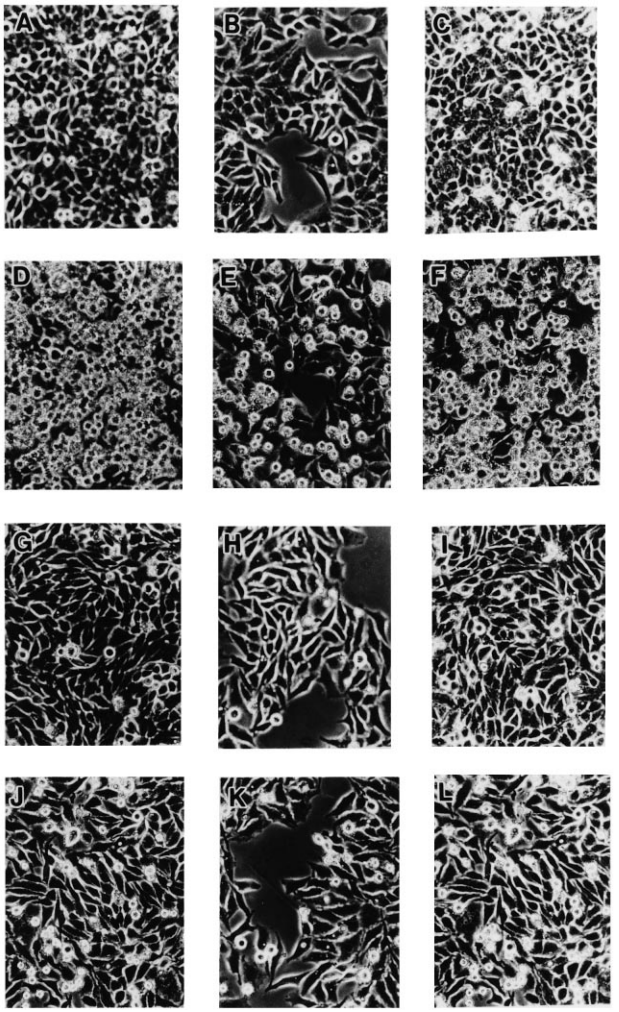

Fig. 3. Morphological apoptotic changes of HEp-2 cells. HEp-C (A–F) and HEp-R (G–L) cells were cultured for 1 h in the absence (A–C and G–I) or presence of sorbitol (0.3 M) (D–F and J–L), and then cultured in sorbitol-free medium for 1 h as previously described [19]. Photographs of various microscope fields were taken.

here that a faint amount of Vpr, non-detectable by Western blotting, is enough for anti-apoptotic action. Since clonal selection of cells other than G418 resistance was not carried out in our study described here, the anti-apoptotic nature of HEp-R cells should be a general property of Vpr-expressing cells.

Our findings described here have important biological implications. The virion protein Vpr can interfere with the physiological turnover of cells at an early phase of infection, and thus can facilitate virus persistence among cells. This anti-apoptotic effect of Vpr may also result in the slight enhancement of virus replication in cells as previously reported for HSV-1 [19], and subsequently, virus spread in an individual. Reports by us and others are consistent with this hypothesis [5,25].

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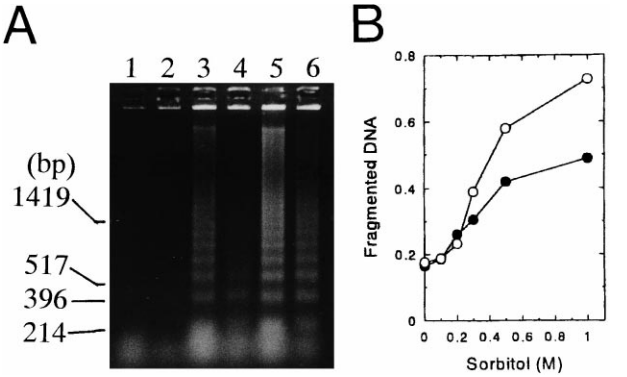

Fig. 4. Biochemical apoptotic changes of HEp-2 cells. [^{3}H]Thymidine-labeled HEp-C (lanes 1, 3, and 5 in A) and HEp-R (lanes 2, 4, and 6 in A) cells were incubated in medium containing sorbitol (lanes 1 and 2, without sorbitol; lanes 3 and 4, 0.3 M sorbitol; lanes 5 and 6, 0.5 M sorbitol) for 1 h, and then incubated in medium without sorbitol for an additional 1 h as previously described [19]. Fragmented DNA was extracted, and analyzed in a 1.5% agarose gel (A) as previously described [19,20]. For quantitation, radioactivities in fractions of total and fragmented DNA were determined and the ratio was calculated (B) as previously described [19,20]. Symbols in B: open circles, HEp-C cells; closed circles, HEp-R cells.

References

[1] Levy, J.A. (1993) Microbiol. Rev. 57, 183–289.
[2] Cullen, B.R. and Greene, W.C. (1989) Cell 58, 423–426.
[3] Trono, D. (1995) Cell 82, 189–192.
[4] Miller, R.H. and Sarver, N. (1997) Nature Med. 3, 389–394.
[5] Ogawa, K., Shibata, R., Kiyomasu, T., Higuchi, I., Kishida, Y., Ishimoto, A. and Adachi, A. (1989) J. Virol. 63, 4110–4114.
[6] Balotta, C., Lusso, P., Cowley, R., Gallo, R.C. and Franchini, G. (1993) J. Virol. 67, 4409–4419.
[7] Kawamura, M., Ishizaki, T., Ishimoto, A., Shioda, T., Kitamura, T. and Adachi, A. (1994) J. Gen. Virol. 75, 2427–2431.
[8] Connor, R.I., Chen, B.K., Choe, S. and Landau, N.R. (1995) Virology 206, 935–944.
[9] Tokunaga, K., Ishimoto, A., Ikuta, K. and Adachi, A. (1997) Arch. Virol. 142, 177–181.
[10] He, L., Choe, S., Walker, R., Di Marzio, P., Morgan, M.D. and Landau, N.R. (1995) J. Virol. 69, 6705–6711.
[11] Jowett, J.B.M., Planelles, V., Poon, B., Shah, N.P., Chen, M.-L. and Chen, I.S.Y. (1995) J. Virol. 69, 6304–6313.
[12] Re, F., Braaten, D., Franke, E.K. and Luban, J. (1995) J. Virol. 69, 6859–6864.
[13] Rogel, M.E., Wu, L.I. and Emerman, M. (1995) J. Virol. 69, 882–888.
[14] Levy, D.N., Fernandes, L.S., Williams, W.V. and Weiner, D.B. (1993) Cell 72, 541–550.
[15] Stewart, S.A., Poon, B., Jowett, J.B.M. and Chen, I.S.Y. (1997) J. Virol. 71, 5579–5592.
[16] Ayyavoo, V., Mahboubi, A., Mahalingam, S., Ramalingam, R., Kudchodkar, S., Williams, W.V., Green, D.R. and Weiner, D.B. (1997) Nature Med. 3, 1117–1123.
[17] Conti, L., Rainaldi, G., Matarrese, P., Varano, B., Rivabene, R., Columba, S., Sato, A., Belardelli, F., Malorni, W. and Gessani, S. (1998) J. Exp. Med. 187, 403–413.
[18] Yao, X.-J., Mouland, A.J., Subbramanian, R.A., Forget, J., Rougeau, N., Bergeron, D. and Cohen, E.A. (1998) J. Virol. 72, 4686–4693.
[19] Koyama, A.H. and Miwa, Y. (1997) J. Virol. 71, 2567–2571.
[20] Koyama, A.H. and Adachi, A. (1997) J. Gen. Virol. 78, 2909–2912.
[21] Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A. and Martin, M.A. (1986) J. Virol. 59, 284–291.
[22] Nishino, Y., Myojin, T., Kamata, M. and Aida, Y. (1997) Biochem. Biophys. Res. Commun. 232, 550–554.

[23] Willey, R.L., Smith, D.H., Laskey, L.A., Theodore, T.S., Earl, P.L., Moss, B., Capon, D.J. and Martin, M.A. (1988) J. Virol. 62, 139–147.
[24] Koyama, A.H. (1995) Virus Res. 37, 285–290.
[25] Lang, S.M., Weeger, M., Stahl-Henning, C., Coulibaly, C., Hunsmann, G., Muller, J., Muller-Hermelink, H., Fuchs, D., Wachter, H., Daniel, M.M., Desrosiers, R.C. and Fleckenstein, B. (1993) J. Virol. 67, 902–912.