Human immunodeficiency virus infection in vitro activates naturally integrated human papillomavirus type 18 and induces synthesis of the L1 capsid protein

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Introduction

Epidemiology indicates a clear link between human immunodeficiency virus (HIV)-positive individuals. To highlight the effect of HIV on HPV expression, HPV-18-positive HIV-permissive HeLa-T4 cells were either infected with HIV-1 or treated with Tat or with the cytokines IL-1α, IL-1β, IL-6 and TNF-α. The presence of HPV-18 E1 (early) and L1 (late) transcripts was then determined by dot-blot or Northern blot hybridization with E1 and L1 or with genomic HPV-18 DNA probes, respectively. Protein extracts from parallel cultures were challenged by Western blotting with an antiserum raised against an L1–β-galactosidase hybrid protein. Results indicated that HeLa-T4 cells constitutively express E1 and L1 transcripts. When cells were infected with HIV, the amounts of E1 and L1 RNAs increased with time, followed by the de novo appearance of L1 protein. E1 and L1 transcripts were also increased, in a dose-dependent manner, by treatment of uninfected cultures with Tat or with IL-6, but were not affected by IL-1α, IL-1β and TNF-α. Neither Tat nor IL-6 could induce L1 translation. These findings raise the hypothesis that the increase of HPV shedding and of HPV-associated diseases in HIV-infected individuals could be due in part to a direct or cytokine-mediated action of HIV, in addition to the HIV-induced immunodeficiency.
detected in all layers of the infected epithelium. Late transcription, the synthesis of L1 and L2 capsid proteins and the production of infectious virus are restricted to the terminally differentiated keratinocytes of the upper layers, close to the epithelial surface (Flores & Lambert, 1997; Frattini et al., 1997; Meyers et al., 1997). Late transcripts are also detected in lower layers, but they remain in the nucleus, due to a block in RNA processing (Stoler et al., 1992). In fact, an AU-rich, cis-acting inhibitory sequence that is found in HPV late mRNAs binds to cellular proteins and is probably involved in the inhibition of HPV late gene expression in undifferentiated epithelial cells (Zhao et al., 1996). In HeLa cells, as well as in other cervical carcinoma cells, only the transcription of early HPV genes was detected, at least in the poly(A)⁺ RNA fraction (Hummel et al., 1995; Inagaki et al., 1988; Schwarz et al., 1985).

The aim of this study was to highlight the effect of HIV infection on HPV-18 gene expression in epithelial cells, and to explore the possibility of a direct stimulation of HPV by HIV superinfection. HPV-18-positive HeLa-T4 cells were exogenously exposed to HIV-1, to the HIV transactivator Tat or to cytokines induced by HIV. The E1 and L1 genes were selected, as representative of HPV-18 expression. The former encodes a phosphoprotein that provides several DNA replication functions (Melendy et al., 1995), while the latter encodes the major component of the HPV capsid. The data indicate that there are increased levels of E1 and L1 HPV-18 transcripts, followed, only in HPV-infected epithelial cells, by a de novo synthesis of the HPV L1 protein.

Methods

■ Cells. HeLa-T4 epithelial cells were obtained from the MRC AIDS-directed Programme Reagent Project (NIBSC, UK). These cells are derived from a human cervical carcinoma, contain 10–50 copies of HPV-18 sequences (Schwarz et al., 1985) and are CD4-transfected. Cultures were maintained as in Dolei et al. (1998).

■ HIV-1. The T-tropic isolate HIV-1ép, was used. Virus characteristics and stock production have been described (Dolei et al., 1998). Infection of HeLa-T4 cells was done as in Dolei et al. (1994). The m.o.i. was 0.1–0.5 syncytium-forming units (s.f.u.) per cell. At various time-intervals, cell culture supernatants and washed monolayers were collected and then incubated at ~80 °C. Virus replication was evaluated as infectious virus, as described (Dolei et al., 1994; Toniolio et al., 1995).

■ Cytokine detection. Culture fluids received 0·5% Triton X-100 to inactivate infectious HIV. To detect IL-1α, IL-1β, IL-6 and TNF-α, an ELISA assay (Medgenix Diagnostics, Fleursus, Belgium) was carried out, according to the manufacturer’s instructions.

■ Cytokine and Tat treatment. Human recombinant IL-1α, IL-1β, IL-6 and TNF-α (Boehringer Mannheim), and HIV-1 Tat (E. coli-derived HxB2 Tat; Intracell, London, UK) were added to cultures 24 h after seeding, at the following concentrations: 20 U/ml (IL-1α, IL-1β); 10–1000 U/ml (IL-6); 20 pg/ml (TNF-α); 0·1–100 ng/ml (Tat) (and maintained throughout the experiments).

■ RNA extraction. Total cellular RNA was obtained as described (Chomczynski & Sacchi, 1987). To demonstrate that RNA extracts were free of contaminating DNA, aliquots of each RNA sample were treated with RNase prior to hybridization: this treatment abolished the positive signals obtained in untreated samples hybridized to specific probes. RNA concentrations in the preparations were determined as absorbance at 260 nm.

■ Preparation of E1-, L1- and β-actin-specific probes. A pGEM-2 plasmid containing the whole HPV-18 genome inserted in the EcoRI site and human cellular DNA (containing the β-actin gene) were used to prepare the radiolabelled probes. The E1, L1- and β-actin-specific probes were prepared by PCR utilizing primers and procedures as described (Bernard et al., 1994; Contorni & Leoncini, 1993). PCR reactions for each set of primers were performed separately in a total volume of 100 µl containing 1 ng of HPV-18 recombinant plasmid DNA (or 1 µg of cellular DNA, as for β-actin amplification) and 50 µCi [α-³²P]dATP. The amplified products (probes) were gel-filtered through a QiAquick column to remove unincorporated nucleotides.

■ Dot-blot hybridization. Aliquots of cellular RNA (1 µg) were mixed with 3 vols of 10 × SSC, 0·1 M formaldehyde and denatured by heating at 65 °C for 5 min. Each RNA sample was spotted onto three nitrocellulose membranes (Hybond-C extra; Amersham), to be hybridized to the E1, L1 and β-actin probes, essentially as in Sambrook et al. (1989). The hybridization was carried out overnight at 42 °C and then excess free probe was washed off (stringent conditions: two washes with 0·1% SDS, 2 × SSC at room temperature and a final wash with 0·1% SDS, 0·2 × SSC at 50 °C). The membranes were air-dried at room temperature and specific hybridization was evaluated as radioactivity bound to the membranes (quantified by scanning the spots with an Instant Imager Electronic Autoradiography apparatus [Packard]. Background radioactivity was subtracted from the crude spot values and data obtained with the E1 and L1 probes were then normalized with respect to those obtained with the same samples hybridized with the β-actin probe, and therefore expressed as a percentage of the β-actin counts. The latter gene was chosen as housekeeping gene since its expression has been shown to remain unchanged in cells infected with HIV or exposed to Tat (Fan et al., 1997; Ito et al., 1998). Since the E1, L1 and β-actin probes were prepared separately, they differed in specific radioactivity: thus the data obtained for E1 and L1 expression cannot be quantitatively compared each other. The statistical analysis was based on the non-parametric paired Wilcoxon’s test.

■ Northern blot hybridization. Sixty µg of total RNA extracted from HeLa-T4 cells 2 days after HIV infection and from parallel control cultures was electrophoresed through 1 × MOPS buffers, 16·5% formaldehyde (37%, v/v), transferred to nitrocellulose membranes for 2 h at 60 mA, as described (Sambrook et al., 1989), hybridized with the complete genomic HPV-18 DNA probe, and scored as described above for dot-blot hybridization. The HPV-18 genomic DNA was purified from a pGEM-2 vector, after EcoRI digestion, by agarose gel electrophoresis and was labelled by nick-translation as described (Schwarz et al., 1985).

■ Western blotting. Parallel cultures of HeLa-T4 cells were washed, lysed in 0·5% Triton X-100 and assayed for protein content by the bicinchoninic acid assay (Sigma). Fifteen µg of protein from each sample was resuspended in loading buffer (50 mM Tris–HCl, pH 6·8, 2% SDS, 35 mM β-mercaptoethanol, 10% glycerol and 0·1% bromophenol blue), loaded onto 10% SDS–polyacrylamide gels, and electrophoresed for 60 min at 60 mA, as described (Sambrook et al., 1989). Samples were then transferred onto nitrocellulose membranes (Hybond-C extra, Amersham) with a semi-dry blotter apparatus (KEM EN TECH) for 2 h at 47 V and 180 mA. Membranes were challenged with a polyclonal rabbit antiserum kindly provided by S. Campo, Beatson Institute, Glasgow, UK. This
Results

HIV replication and cytokine induction

HeLa-T4 cells are highly permissive for HIV-1 and release high titres of infectious virus (Dolei et al., 1994). Cells were tested for production of four cytokines, selected because they are relevant for HIV replication and spread (IL-1α, IL-1β, IL-6, TNF-α; Fauci, 1996). In Table 1 data are presented on cytokine production 48 h after HIV-1P1 infection. Of particular note is the induction of IL-6 and TNF-α, which stimulate HIV replication in circulating (Fauci, 1996) and adherent cells (Dolei et al., 1994, 1996). Treatment of parallel cultures with HIV Tat 100 ng/ml for 48 h ) induced cytokine levels approximately similar to those induced in the same cells by an HIV inoculum of 0.05 s.f.u. per cell (not shown).

Expression of HPV-18 genes: stimulation by HIV infection

HeLa-T4 cells were kept in culture either without additional treatment or infected with HIV-1P1; at various times samples were collected for quantification of HPV-18 expression, as indicated in Methods. Results of dot-blot hybridizations (net counts) were normalized to the values for expression of the constitutive β-actin gene, whose expression is unaffected in cells infected by HIV or exposed to Tat (Fan et al., 1997; Ito et al., 1998). Data in Fig. 1 represent the average of six separate experiments, with independent labelling of the probes, and indicate that HeLa-T4 cells constitutively express HPV-18 E1 and L1 RNA. Subsequent to cell subcultivation, a burst of both transcripts is observed, reaching a maximum 24 h after seeding and returning almost to base levels after 72 h. When the cells are infected by HIV-1 there is a statistically significant increase in accumulation of HPV transcripts, maximal 24 h after infection for E1 and 48 h after for L1. Seventy-two hours after HIV infection the accumulation of E1 and L1 transcripts is 7- and 2.5-fold, respectively, that of control cultures; differences in transcript stability may account for the lower accumulation

Table 1. Response of HeLa-T4 cells to HIV-1 infection: virus yields and cytokine production

<table>
<thead>
<tr>
<th></th>
<th>log s.f.u. HIV</th>
<th>IL-1α (pg)</th>
<th>IL-1β (pg)</th>
<th>IL-6 (pg)</th>
<th>TNF-α (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>0.8</td>
<td>0.4</td>
<td>151.8</td>
<td>0.5</td>
</tr>
<tr>
<td>HIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m.o. 0.05</td>
<td>4.9</td>
<td>1.9</td>
<td>1.8</td>
<td>1005.6</td>
<td>15.4</td>
</tr>
<tr>
<td>m.o. 0.5</td>
<td>5.8</td>
<td>3.6</td>
<td>2.6</td>
<td>2614.7</td>
<td>38.1</td>
</tr>
</tbody>
</table>
of L1 transcripts (Hummel et al., 1995), probably determined by the AU-rich, cis-acting inhibitory sequence of HPV late mRNAs (Zhao et al., 1996). In Fig. 1 the relative expression of HPV-18 E1 or L1 with respect to the β-actin gene seems to decrease with time: this means that the transcription of HPV-18 E1 and L1 genes during the early phases of cell growth is higher than that of the β-actin gene, which is considered to be invariant with time; absolute amounts of HPV-18 transcripts, however, increase with time.

To analyse whether HIV infection could qualitatively change HPV-18 transcription, total RNA accumulated within 48 h in untreated and HIV-infected cells was isolated and subjected to Northern blot hybridization with a total HPV-18 DNA probe, as described in Methods. Data (Fig. 2) for both experimental conditions show the same RNA pattern, consisting of two major RNA species, approximately 1.5 and 3.5 kb, that are more abundant in HIV-infected cultures.

Transcriptional effects of Tat and of HIV-induced cytokines

Experiments were carried out in order to evaluate whether HIV transcriptional effects on HPV-18 genes were due to a direct action of HIV, through its trans-activating protein Tat, or whether they were mediated by the induction of cellular products, such as proinflammatory cytokines.

Cells were therefore treated with graded amounts of recombinant HIV Tat. As shown in Fig. 3(A, B) exogenous Tat exerts a transcriptional effect on the HPV-18 E1 and L1 genes, and this action is dose dependent. This finding demonstrates a direct action of HIV on HPV-18 activation. The effect is seen as early as 24 h after treatment, while differences with controls are less pronounced after more prolonged incubations (data not shown).

Since HIV-infected HeLa-T4 cells produce some proinflammatory cytokines, which enhance further HIV spread (Dolei et al., 1994, 1996), cultures of HeLa-T4 cells were treated with IL-1α, IL-1β, IL-6 and TNF-α. These cytokines are usually detected in HeLa-T4 cells after HIV infection (Table 1), and the concentrations used were in the range of those induced by HIV. The data obtained after treating cultures with graded amounts of IL-6 are shown in Fig. 3(C, D). Exposure of cultures to this cytokine resulted in a dose-dependent increase in transcription of both E1 and L1 genes. At variance with the increase produced by Tat, IL-6 effects on HPV-18 transcription increased with time; the data reported in Fig. 3(C, D) were obtained after 3 days of incubation. IL-6 effects on E1 were detectable as early as 24 h while the L1 increase started from day 2 onward (data not shown). Neither TNF-α nor IL-1α nor IL-1β had any reproducible effect on E1 and L1 transcription, at...
Fig. 4. HIV-induced translation of L1 major capsid protein in HeLa-T4 cells. HeLa-T4 cells were either infected with HIV or treated with Tat or the cytokines IL-6 and TNF-α. Cell lysates, obtained as described in Methods, were challenged in Western blot assays with an HPV-specific antiserum recognizing the L1 major capsid protein. Upper panels, Western blotting membranes; lower panels, quantification of specific bands present on the membranes by scoring in an image analyser. Panels (A) and (C), time-course of L1 protein accumulation during HIV infection, and lack of effect of IL-6; panels (B) and (D), L1 protein detection at day three of culture in HIV-infected HeLa-T4 cells only, and lack of effect of Tat, IL-6 and TNF-α. Controls included the buffer and lysates from the unrelated HIV-infected cell line C8166 (negative controls), and the recombinant L1–β-galactosidase hybrid protein (positive control). Values in (C) and (D) are not quantitatively comparable, since the two membranes had different backgrounds.

least at the concentrations used and within 3 days of treatment (data not shown).

HIV-induced translation of L1 major capsid protein

Lysates from parallel cultures of HeLa-T4 cells were challenged in Western blot assays with an HPV-specific antiserum recognizing the L1 major capsid protein. Fig. 4 shows the data obtained after HIV infection (A, C) and after a 3 day treatment with Tat or the inflammatory cytokines IL-6 and TNF-α (B, D). The blots themselves (A, B) and the quantification of specific bands, obtained by scoring with an image analyser (C, D), are shown. HeLa-T4 cells alone did not produce any detectable L1 protein. However, after HIV infection (arrows in A), a band recognized by the HPV L1-specific antiserum appeared and accumulated with time. Western blotting of lysates from cells treated with Tat, IL-6 or TNF-α did not show any L1 band (B). The specificity of the serum employed was demonstrated by its recognition of a recombinant L1–β-galactosidase hybrid protein (Fig. 4B), thereby excluding any possible cross-reactivity with a cellular or viral protein of similar size, e.g. HIV p55\textsuperscript{p24}. Lysates from untreated and HIV-infected C8166 cells were challenged in Western blot with the HPV-specific serum, and did not show any staining.

Discussion

The present study shows that HIV-1 infection of HPV-18-bearing epithelial cells results in activation of both E1 (early) and L1 (late) HPV-18 genes. These findings have not been reported previously, and are in keeping with reports of increased HPV infection and pathogenicity in HIV-infected individuals (Palefsky, 1997; Six et al., 1998; Sun et al.,...
et al. (1994) and Dolei et al. (1996). Apart from the immunodeficiency resulting from HIV infection, this might be caused by a direct action of HIV on HPV-infected cells: epithelial cells may host HIV replication in vitro and in vivo (Heng et al., 1994; Dolei et al., 1996; Fauci, 1996; Furuta et al., 1994; Toniolo et al., 1995), although to a limited extent, and, more frequently, may be exposed to HIV products, such as Tat, and to HIV-induced cytokines (Mostad & Kreiss, 1996; Iversen et al., 1998; Vernon et al., 1994; Goulston et al., 1998). Therefore, we infected HPV-18-bearing HeLa-T4 cells with HIV-1 in an attempt to add to the results obtained by transfection techniques and the use of constructs (Vernon et al., 1993; Tornesello et al., 1993; Barillari et al., 1992; Kyo et al., 1994; Tan et al., 1995). In comparison with the studies reported so far, our experimental model is closest to the situation in vivo (Vernon et al., 1994): in fact HeLa cells are derived from a HPV-18 infection in vivo, and were exogenously infected with HIV-1 in a natural way, through binding to cell receptors and normal entry.

We observed basal transcription of both E1 and L1 in untreated HeLa-T4 cells. The latter finding is notable, since late gene expression is normally restricted to terminally differentiated keratinocytes (Flores & Lambert, 1997; Frattini et al., 1997; Meyers et al., 1997). One must recall, however, that we analysed total cellular RNA, thus including nuclear transcripts. When HeLa-T4 cells are infected by HIV-1, HPV-18 transcripts accumulate to levels 2- to 7-fold greater than in control cultures. HIV infection, however, does not change qualitatively the pattern of HPV-18 transcription (Schwarz et al., 1985), as indicated by Northern blot hybridization, but transcripts are more abundant in HIV-infected cultures (Fig. 2). The accumulation of HPV-18 RNAs could be due either to an increased transcription rate or to reduced degradation of E1 and L1 transcripts in HIV-infected cells. It is unlikely that a direct activation of specific cellular flanking genes could be responsible for HPV-18 activation, since HPV-18, as well as HPV-16, is integrated into several chromosomes (Popescu et al., 1987; Jeon et al., 1995).

The importance of the HIV Tat and Rev regulatory proteins is well known, both in vivo and in vitro; HIV expression is under the control of two modulatory mechanisms, the Tat/TAR (Tat activation response) and Rev/RRE (Rev-responsive elements) pathways; these are linked to the endogenous cytokine network, and to possible interactions with other co-infecting agents [reviewed in Levy (1994) and Fauci (1996); see also Felber et al., 1990]. Tat is a potent transactivator of transcription of both viral and cellular genes, through interactions with the TAR RNA elements and cellular proteins (Kao et al., 1987; Levy, 1994; Yang et al., 1997). In vivo, biologically active Tat can be released by HIV-infected cells into circulating body fluids (Ensoli et al., 1994), and both in vivo and in vitro may be captured by several uninfected cell types, through binding to integrin receptors, and transactivate cellular promoters as well as homologous and heterologous viral promoters (Levy, 1994; Harrington et al., 1997). Rev acts post-transcriptionally on viral and cellular mRNAs containing Rev-responsive sequences, both to prevent nuclear degradation of pre-mRNAs and to shuttle them from the nucleus to the cytoplasm (Levy, 1994; Malim & Cullen, 1993). In addition, HIV infection promotes the release of several cytokines, especially IL-6 and TNF-α (Table 1), as shown for various cell systems, (Levy, 1994; Dolei et al., 1994 1996; Fauci, 1996); these cytokines have been shown to stimulate HIV replication in circulating (Fauci, 1996) and adherent cells (Dolei et al., 1994, 1996).

In patients infected by both HIV and HPV, HPV-bearing epithelial cells are exposed not only to HIV virions, but also to soluble viral and cellular factors released within the tissue both by resident HIV-infected cells or by HIV-infected infiltrating cells, especially Tat and pro-inflammatory cytokines (Levy, 1994; Fauci, 1996). We therefore exposed HeLa-T4 cells to Tat or to HIV-inducible cytokines in our experimental system. Exposure to Tat resulted in dose-dependent increased transcription/accumulation of E1 and L1 genes, thus indicating that naturally integrated HPV genes are sensitive to Tat transactivation. Other workers have reported (results obtained by transfection of constructs) increased E2-dependent HPV-16 transcription (Vernon et al., 1993), and HPV long control region transactivation and HPV-18 E7 expression (Tornesello et al., 1993); in contrast, Gius & Laimins (1989) did not find Tat effects studying an HPV-18 promoter-containing plasmid. On the other hand proinflammatory cytokines, whose production intended to contribute to the eradication of virus infection, can promote HIV gene expression and interrupt latency by rescuing defective HIV provirus and increasing Tat production (Levy, 1994; Fauci, 1996; Barillari et al., 1992), virus release and binding to uninfected cells (Dolei et al., 1994, 1996). There are only two reports on cytokine regulation of HPV early gene expression: (i) a downregulation by IL-1 and by TNF-α (with no effect of IL-6) of HPV-16 early genes (by transfection of the HPV-16 noncoding region in HPV-18-positive HeLa cells; Kyo et al., 1994); (ii) HPV-16 E6 and E7 downregulation by IL-1α and TNF-α in cervical carcinoma cells (Woodworth et al., 1995). Of the cytokines produced by naturally infected HeLa-T4 cells after HIV infection (IL-1α, IL-1β, IL-6 and TNF-α), in our experimental conditions, no downregulation of E1 and L1 RNAs by IL-1α, IL-1β and TNF-α was observed, while IL-6 stimulated in a dose-dependent manner the transcription/accumulation of both genes (Fig. 3C, D), as it does also for HIV genes (Levy, 1996; Dolei et al., 1994, 1996). Effects of IL-6 were detectable as early as after 24 h (E1) and 48 h (L1, increasing with time.

An interesting finding of the present work is the detection of L1 protein in HIV-infected HeLa-T4 cells. This was believed to be synthesized only in terminally differentiated keratinocytes of the upper layers (Frattini et al., 1997), due to a block in late RNA processing (Stoler et al., 1992; Zhao et al., 1996). Unexpectedly, HIV infection of HeLa-T4 cells bypassed this
inhibition, as indicated by the accumulation of HPV-18 L1 protein with time during HIV replication (Fig. 4), and this confirms that HIV can disrupt the tight regulation of HPV transcription and translation. In keeping with our results, obtained with a more natural system, are the data from Tan et al. (1995) and Tan & Schwartz (1995) who, by cell coinfection with constructs containing HIV and HPV genes, showed that Rev expression by epithelial cells counteracts the effect of the AU-rich negative element in HPV late mRNAs. This helps to explain why in our hands cell exposure to Tat activates only HPV transcription, while HIV infection (with endogenous production of Rev) is required for L1 protein translation. From these results of ours and others, it may be concluded that the mechanism of HPV induction by HIV could be as follows. HIV activation of HPV transcription occurs directly through Tat and indirectly through IL-6 induction, followed by late HPV translation, due to Rev shuttling of mRNA and counteraction of the AU-rich negative element in the HPV late 3' untranslated region. Moreover, published data show that in vivo HIV infects herpes simplex virus-1-infected epidermal keratinocytes, with reciprocal enhancement of replication (Heng et al., 1994). This might be a more general phenomenon, raising the possibility that HPV infection could render keratinocytes more susceptible to HIV, therefore entering a vicious circle of synergistic expression of the two viruses. In this respect, the finding of early regression of HPV lesions in HIV-infected women responding to antiretroviral therapy (Heard et al., 1998) is relevant.

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References


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