Antagonistic modulation of human cytomegalovirus replication by transforming growth factor β and basic fibroblastic growth factor

J. Alcami,† C. V. Paya,‡ J. L. Virelizier and S. Michelson*

Unité d’Immunologie Virale, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cédex 15, France

We studied the effect of two cytokines, basic fibroblastic growth factor (bFGF) and transforming growth factor beta (TGF-β) on human cytomegalovirus (HCMV) replication in cultured human lung fibroblasts. We show that TGF-β increases HCMV production, probably by a transcriptional mechanism, and that bFGF represses HCMV replication in a dose-dependent manner. These actions were antagonistic and the mechanisms involved were independent of the effects of these factors on cell DNA synthesis and proliferation.

Replication of human cytomegalovirus (HCMV) is highly dependent on the state of cellular activation and the cell cycle. It has been found that the bulk of HCMV DNA synthesis occurs when no cellular DNA synthesis is detectable (reviewed in Albrecht et al., 1989). Moreover, blocking of host DNA synthesis has been reported to increase both permissiveness to infection and the rate of HCMV replication (St Jeor et al., 1974). Cell cycling is governed by complex interactions between growth factors. Among these factors, basic fibroblastic growth factor (bFGF) and transforming growth factor beta (TGF-β) have opposite effects on fibroblast proliferation in culture (reviewed in Roberts & Sporn, 1988). Furthermore, both factors can be produced either by cells infected with HCMV (Alcami et al., 1991) or by other cells during the course of immune and inflammatory responses to infection (Kehrl, 1986; Assoian et al., 1987; Wahl et al., 1987). Therefore, in order to gain a better understanding of HCMV host–cell relations and mechanisms of viral latency and reactivation, we have examined the effect of these two cytokines on viral replication in infected human fibroblasts (MRC-5).

To study the role of cytokines on viral replication it was necessary to develop culture conditions without serum, otherwise the presence of traces of the cytokines studied might interfere with the experimental results. We have previously developed and described elsewhere (Alcami et al., 1991) a method of fibroblast culture in the absence of serum that does not interfere with HCMV replication. Briefly, human diploid lung fibroblasts (MRC-5; Jacobs et al., 1970) grown in Dulbecco’s modified medium (D) supplemented with 10% foetal calf serum (FCS), 10 mM-Tricine pH 7-6 and 0.5% bicarbonate were seeded at 10^5 cells/well in 24-well plates. Following overnight incubation, cells were washed twice with PBS and transferred to serum-free medium supplemented with human transferrin (T) (10 mg/ml, Sigma) and bovine insulin (I) (2.5 mg/ml, Sigma), designated D+T+I, and incubated for 48 h. Cells were then infected with the AD169 strain of HCMV at 5 p.f.u./cell with a 1 h adsorption period at 37 °C. Mock-infected cells were incubated with medium containing an amount of serum equivalent to that in the virus inoculum. Cells were washed twice with PBS and refed in D+T+I with or without growth factors. Supernatants collected at different times were clarified at 2000 r.p.m. for 10 min at 4 °C, and stored at −80 °C until titration by plaque formation. Under these conditions, titres of virus were lower, by an average of log 1.6, than virus production in the presence of 5% serum in the same experiment (results not shown). To determine whether the growth factors at the concentrations used during titration could affect titres, we added T+I to a normal stock of virus and either bFGF (5 ng/ml) or TGF-β (2 ng/ml) before diluting stepwise to 10^-3 (highest concentrations used during titration). No significant differences were found (T+I alone = 8.3±1.2x10^4; +TGF-β = 7.0±8x10^4; +bFGF = 6.3±0.5x10^4).

Under these culture conditions, addition of purified platelet-derived TGF-β (British Biotechnology, U.K.) after viral adsorption at doses ranging from 0.1 to 2 ng/ml resulted in increased viral production (Fig. 1a) which was significantly elevated at the two highest doses. As expected, TGF-β inhibited cellular DNA synthesis as measured by thymidine incorporation (Fig. 1b). Recombinant bFGF (a kind gift from Professor W. Fiers, Gent, Belgium) at doses of 0.1 to 5 ng/ml had the opposite effect,
Fig. 1. Effect of purified TGF-β on HCMV replication and thymidine incorporation in fibroblasts. (a) Extracellular virus production in the presence of different concentrations of TGF-β. Serum-deprived human lung MRC-5 fibroblasts were infected with HCMV at 5 p.f.u./cell for 1 h and refed in D+T+1 medium with different concentrations of TGF-β. Media were collected at 5 days p.i. and titrated by plaque assay. Results are expressed as mean p.f.u./ml in three wells and correspond to one of three experiments. Standard deviations are represented by thin bars over each column. Significant differences are: *** < 0.005; ** < 0.02; * < 0.05; (.) < 0.1 and > 0.05. (b) [3H]-Thymidine incorporation (1 μCi/ml) into non-infected, serum-deprived fibroblasts from 0 to 24 h after mock infection. Cells were treated with TGF-β as described above. At the end of the labelling period, cells were maintained on ice and washed in situ with PBS, twice with cold 5% TCA at 10 min intervals, then once with PBS before being solubilized in 0.5 ml of 100 mM-Tris-Cl pH 7.4, 1 mM-EDTA and 0.1% SDS. An aliquot (0.25 ml) of each sample was counted in ACS (Amersham) scintillation liquid.

significantly diminishing viral replication in a dose-dependent manner (Fig. 2a) and enhancing thymidine incorporation (Fig. 2b), except at the highest concentration (5 ng/ml).

To determine whether these effects were associated with an increase in viral DNA synthesis, cells (5 x 10⁶) were seeded and cultured in the absence of serum before infection, and with or without growth factors after infection. DNA extraction was done 5 days post-infection (p.i.) according to the Hirt technique (Hirt, 1967) and low M₅ DNA was hybridized with the AD169 HindIII X fragment excised from plasmid HAD-23 (kindly provided by R. Colimon, Laboratoire de Bacteriologie-Virologie, Rennes, France). Viral DNA was spotted at different concentrations on a nylon membrane (Hybond N, Amersham) as described by Davis et al. (1986) and the filter was hybridized with a probe labelled by random-priming according to the manufacturer's instructions (Boehringer-Mannheim). As shown in Fig. 3(c) viral DNA synthesis was slightly increased in infected cells treated with either bFGF (5 ng/ml) or TGF-β (2 ng/ml) compared to that in cells cultured in medium alone. Nevertheless, no correlation was seen between viral DNA synthesis and HCMV virion production in bFGF-treated cells, thus suggesting that the negative effect of this cytokine may be exerted at a late stage of development (virion assembly and maturation). No hybridization was seen with low M₅ DNA extracted from uninfected cells, whether or not they were treated with cytokines (results not shown).

An inverse correlation between cellular DNA synthesis and viral replication has been reported (see Albrecht et al., 1989). In the light of this and because both cytokines affect cell proliferation, we determined whether the effects of bFGF and TGF-β on HCMV replication were secondary to their effects on cell growth. For this purpose, we analysed the effects of both cytokines on
Fig. 3. Effect of TGF-β (a) and bFGF (b) on HCMV production from irradiated cells. Production of extracellular HCMV from cells irradiated (5000 rads) 4 h before virus adsorption and treated as described in Fig. 1. Results shown are representative of three experiments. Bars indicate standard deviations and asterisks correspond to levels of significance as defined in the legend of Fig. 1. (c) A semi-quantification of CMV DNA in infected cells treated by (A) 5 ng/ml of bFGF (B) D + T + I alone or (C) 2 ng/ml of TGF-β. Different quantities of DNA were hybridized with the HindIII X fragment of the AD169 strain of HCMV.

HCMV replication in cells in which DNA synthesis had been arrested by gamma irradiation (5000 rads, 4 h before infection). This radiation reduced thymidine incorporation by more than 90% (not shown). As seen in Fig. 3(a, b), the effects of both growth factors remained unchanged. This is of special interest in the case of TGF-β because it has been shown that its inhibitory effects are produced by blocking the cells in the pre-S phase (Chambard & Pouyssegur, 1988; reviewed in Moses et al., 1990). Thus, the effects of these growth factors on HCMV production appear to be independent of their effects on cell proliferation and are probably due to other mechanisms.

The results obtained so far suggest that the antagonistic effects of bFGF and TGF-β on HCMV replication are probably produced by different mechanisms and that the TGF-β-induced increase in viral production might be due to enhanced viral transcription. We therefore performed transient transfection assays with two different constructs of the HCMV promoter: CMV-enh, containing the major immediate early (IE) HCMV enhancer–promoter sequence, −525 to +97 (Schwartz et al., 1990) and wt760, containing the sequence from −753 to +7, and, as a control, d760 containing the sequence −65 to +7 (wt760 and d760 were generous gifts from M. F. Stinski; Stinski & Roehr, 1985). All were cloned upstream of a luciferase reporter gene and transfected into fibroblasts by the calcium chloride method. After transfection, cells were cultured in the absence or presence of 5% FCS. Whereas bFGF had little effect on promoter activities (Fig. 4a), TGF-β induced the trans-activation of both HCMV enhancer–promoters. Activation of wt760 was greater than that of CMV-enh. As the difference between the two constructs is the presence of NF-1-binding sequences in the wt760 enhancer–promoter (Hennighausen & Fleckenstein, 1986; Jeang et al., 1987), it is probable that these sequences may play a major role in the TGF-β induction of CMV. Interestingly, this effect on wt760 was particularly marked when cells were grown in serum following transfection (Fig. 4b). These results suggest that enhancement of HCMV replication by TGF-β could be, at least partially, to a direct transcriptional effect on the major IE HCMV promoter. TGF-β has been shown to induce the trans-activation of different promoters such as the mouse alpha(2)I collagen gene through induction of the transcription factor NF-1 (Rossi et al., 1988). In addition, TGF-β can induce its own transcription by induction of a nuclear factor that binds to both TGF-β promoters via a target sequence containing a phorbol ester-responsive element (TRE) (Kim et al., 1989, a, b; Birchenall-Roberts et al., 1990). Thus, the transcription factor AP1, which recognizes the TRE, is probably the main factor involved in TGF-β autoregulation (Kim et al., 1989 c). In addition, TGF-β induces phosphorylation of the cyclic AMP-responsive element binding protein (CREB) and induces the expression of the early gene jun-B (Pertovaara et al., 1989; Kramer et al., 1991) without modifying levels of c-fos (Takehara et al., 1987; Chambard & Pouyssegur, 1988). Consensus motifs for AP1, CREB, NF-1 and nuclear factor kappa-B have been described in the HCMV major IE enhancer–promoter, and some of these seem to be functional in HCMV transcription (Kuan-Teh et al., 1987; Hunnighake et al., 1989; Sambucetti et
Fig. 4. Effect of TGF-β and bFGF on the HCMV promoter. (a) MRC5 cells were transfected by the calcium chloride method with 5 µg/10^6 cells of CMV-enh (-525 to +97) or wt760 (Stinski & Roehr, 1985) linked to the luciferase gene, then cultivated with 5% FCS in the absence (white) or presence of TGF-β (8 ng/ml) (dark grey) or bFGF (10 ng/ml) (light grey). Twenty-four hours after transfection cells were lysed and luciferase activity was measured as described (Schwartz et al., 1990). Results are expressed in luminometric (Berthold) units of luciferase activity normalized per mg of protein. Numbers above the columns represent the ‘fold’ amplification in the presence of growth factor with respect to untreated cells. (b) Cells were transfected as above with either the CMV-enh (-525 to +97) (hatched) or wt760 (black) enhancer–promoters or the enhancerless promoter d1760 (grey) (Stinski & Roehr, 1985) linked to the luciferase gene. Following transfection, cells were washed and grown in medium containing only transferrin (10 µg/ml) and insulin (2.5 g/ml) and TGF-β (8 ng/ml) (TGF-β+TI) or in the same medium with 5% FCS (TGF-β+TI+FCS). Luciferase activity was measured and expressed as in (a). Numbers above the columns are the ‘fold’ amplification of enhancer activity relative to the enhancerless promoter d1760.

Our results suggest that a transcriptional effect of TGF-β could be mainly mediated through the NF-1 motif present in the CMV major IE enhancer–promoter, as for the collagen promoter (Rossi et al., 1988). Since the enhancing effect of TGF-β on the HCMV IE promoter was only found when cells were maintained in the presence of serum after transfection (Fig. 4b), this raises the possibility that a serum factor results in a post-translational modification of the factor(s) induced by TGF-β from an inactive to an active form. The other possibility is that serum induces another growth factor that acts in synergy with the one(s) induced by TGF-β.

How can these results be reconciled with the effects of TGF-β on HCMV replication seen in the absence of serum? The results obtained in transfection experiments reflect a limited aspect of the numerous mechanisms involved in HCMV replication in host cells. First, transfection bypasses interactions between the virus and its receptor, which could potentially deliver transduction signals capable of inducing nuclear transcription factors. Indeed, HCMV infection modifies the cell environment and has a global stimulatory effect on host cell metabolism (Boldogh et al., 1990; reviewed in Albrecht et al., 1989), including induction of a bFGF-like factor (Alcami et al., 1991). Second, preparations of HCMV can be contaminated by traces of growth factors, since virus adsorption was performed in the presence of low amounts of serum. Finally, the infective process is by far more complex than mere trans-activation of the HCMV IE enhancer–promoter elements in which only a sub-genomic portion of viral DNA is introduced into the cell in the absence of other components found in virions. Taking everything into consideration, we have nevertheless shown here that the increase in viral replication induced by TGF-β is, at least partially, mediated by a transcriptional mechanism involving increased trans-activation of the major IE HCMV enhancer.

Since interplay between growth factors represents a complex phenomenon and since in our experimental model TGF-β and bFGF probably act through different mechanisms, we studied HCMV replication in cells cultured in the simultaneous presence of both factors. We found that bFGF at high doses partially inhibited viral replication stimulated by TGF-β (Fig. 5). bFGF and TGF-β have opposing effects in many cell systems.
(Roberts & Sporn, 1988; Gospodarowicz et al., 1987). The results of their interactions are a function of fibroblast age (Hill et al., 1986), as well as of the overall concentrations of growth factors in the culture medium (Roberts et al., 1985).

The mechanism(s) whereby bFGF suppresses HCMV replication are not understood but appear not to be transcriptional, at least concerning the HCMV major IE transcription. Baird et al. (1990) and Kaner et al. (1990) both described competitive inhibition of herpes simplex virus type 1 adsorption to cells by bFGF. In our studies, bFGF added after virus adsorption would not interfere with this stage of infection. Down-regulation of HCMV replication by bFGF might be related to the fact that this cytokine commits the cell to DNA synthesis and proliferation. One could speculate that entrance into the S phase induced by bFGF (Hall et al., 1991) adversely affects HCMV replication. On the other hand, TGF-β, which arrests cells in the pre-S phase, would create cell environmental conditions more conducive to viral replication. This speculation is supported by increasing evidence that some degree of cell differentiation is probably necessary for productive HCMV infection (Gönczöl et al., 1984; LaFemina & Hayward, 1986; Shelbourn et al., 1989; Ibanez et al., 1991).

The phenomena described here may be relevant to the pathogenesis of HCMV infection in vivo because during inflammatory cell responses these cytokines are produced and could modify both infection kinetics and replication rates. Also, we have described elsewhere that HCMV infection of fibroblasts can induce the production of a factor similar to bFGF (Alcami et al., 1991). Therefore, an equilibrium between host cell factors induced by HCMV infection and, conversely, the influences of bFGF and TGF-β on HCMV replication could be important in explaining some aspects of viral latency and reactivation in the infected host.

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