Induction of apoptosis accelerates reactivation of latent HSV-1 in ganglionic organ cultures and replication in cell cultures

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Herpes simplex viruses replicate at the portal of entry into the body and are transported retrograde to sensory neurons in which they can establish a silent, latent infection characterized by the expression of a noncoding latency-associated transcript and a set of microRNAs. At the portal of entry into the body and in cell culture a viral protein, VP16, recruits cellular proteins that initiate a sequential derepression of several kinetic classes of viral genes. Earlier studies have shown that upon reactivation of latent virus in ganglionic organ cultures all genes are derepressed at once, thus obviating the need for VP16 to initiate sequential derepression of viral genes. One hypothesis that could explain the data is that the massive reactivation of all classes of viral genes is the consequence of activation of an apoptotic pathway. Here we show that two proapoptotic drugs, dexamethasone and 2[[3-(2,3-dichlorophenoxy)propyl]amino]-ethanol, each accelerates viral gene expression in ganglionic organ cultures. We also show that in cultured cells apoptosis induced by dexamethasone accelerates viral gene expression and accumulation of infectious virus. The results are surprising in light of the relatively large number of viral proteins that independently block apoptosis induced by viral gene products or exogenous agents. The results suggest that the virus may rely on apoptosis to exit from latency but that apoptosis may be detrimental for virus replication or spread at the portal of entry into the body.

epidermal growth factor | nerve growth factor | trigeminal ganglia

On entry into cells in culture or at a portal of entry into the body, herpes simplex virus 1 (HSV-1) replicates and ultimately destroys the infected cells. The sequence of events is well defined (reviewed in ref. 1). Thus, upon entry into the nucleus viral DNA is silenced by repressive histones, cellular repressors, and a nuclear body designated ND10 that assembles in contact with the DNA. To replicate, the DNA must be sequentially derepressed (2). At the first checkpoint, a viral protein VP16 or α -transducing factor recruits several cellular proteins but especially Oct1, Host Cell Factor 1 (HCF1), and lysine-specific demethylase 1 (LSD1) to α -gene promoters, enabling the derepression and expression of α -genes (3). Next, at the second checkpoint, one of the α -proteins, ICP0, performs a series of functions that enable derepression of β and some γ -genes (4). These functions include degradation of PML and SP100, dispersal of ND10 bodies, dissociation of HDAC -1 or -2 from CoREST, and ultimately dissociation and export of the CoREST/REST repressor complex to the cytoplasm (5–9). In the last step, viral DNA synthesis enables the expression of the γ_2 (late) gene (reviewed in ref. 1).

In the course of replication at the portal of entry, HSV-1 infects and is transported retrograde to nuclei of sensory neurons. In experimental animal systems, the virus multiplies in some neurons, but in both human and animal systems the virus establishes a latent, silent infection in which the DNA is maintained in episomal form and only the latency-associated transcript and a set of microRNAs (miRNAs) are expressed (10–13, reviewed in ref. 1). In humans, physical stress such as exposure of

skin to UV light or high temperatures, menstruation, or emotional stress induce the virus to multiply and be transported anterograde to a site at or near the site of virus entry into the body (14). On the basis of our current knowledge, the key question regarding the reactivation is the mechanism by which virus replication is initiated because latently infected neurons contain neither VP16 to initiate α -gene expression nor ICP0 to express the β and γ -genes.

The model we have chosen to investigate this process involves organ cultures of murine trigeminal ganglia (TG) harboring latent virus. The TG are removed 30 d after intraocular mouse infection and maintained for 24 h in medium containing anti-NGF antibody. The rationale for this design is threefold. Foremost, the model is based on Cushing's 1905 reports that severance of the connection between the brain and the TG in patients suffering from trigeminal neuralgia resulted in virus reactivation (15). Our model embodies the same design except that the TG are immediately placed in culture medium and incubated at 37 °C. Second, extensive current studies indicate that neurons in peripheral ganglia interact with and both regulate and are regulated by satellite cells that surround them (16, 17). Finally, extensive studies by Wilcox et al. and Camarena et al. (18, 19) have shown that NGF can retard or block viral reactivation. In the model that we have adapted virus reactivation occurs with the time frame of a single cycle of virus replication (2). The key findings in the study of this model were twofold. Foremost, representative α -, β -, γ_1 -, and γ_2 -genes were expressed at once in the absence of protein synthesis from the time of excision of TG. Second, viral gene expression and accumulation of latency-associated transcript (LAT) and viral miRNAs were incompatible and decreased as viral gene product accumulated. The results suggested that reactivation was the consequence of total simultaneous derepression of the viral genome. One hypothesis that could explain this finding is that, in the absence of NGF, neurons initiated a process leading to apoptosis and this resulted in a general derepression of the latently infected cells. In this report, we show that activation of apoptosis in TG maintained in NGF and EGF so as to suppress viral reactivation resulted in accelerated viral gene expression. In cell culture, proapoptotic drugs accelerated viral gene expression. The findings were unexpected inasmuch as herpes simplex virus (HSV) encodes several genes whose function is to block apoptosis induced by exogenous agents or viral gene products.

Relevant to this report are the properties of the two proapoptotic drugs selected for these studies. 2[[3-(2,3-Dichlorophenoxy)propyl]amino]-ethanol (DCPE) induces apoptosis by down-regulating Bcl-XL (20). Dexamethasone has been reported to down-regulate the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-xL, to

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up-regulate the proapoptotic Bak and Bax, and to activate caspases 2 and 3 (21).

Results

Anti-NGF Antibody Accelerates Whereas NGF and Particularly NGF+EGF Delay Reactivation of HSV-1 from TG Organ Cultures. The purpose of the experiments described below was to define the parameters of HSV-1 reactivation in the experiments reported in this article. The TG from mice inoculated 30 d earlier were excised and incubated in medium containing anti-NGF antibody, NGF, or a mixture of NGF+EGF. The TG were individually processed and the mRNAs encoding ICP27 (an α -protein) and VP16 (a γ -protein) were quantified by RT-quantitative PCR (qPCR). In this and all other experiments, each point represents the geometric mean average of six TG processed individually. The results (Fig. 1) show that between 11 and 24 h after excision both ICP27 and VP16 mRNAs increased 10–30-fold. In contrast, the two mRNAs increased barely threefold in medium containing NGF and less than threefold in medium containing both growth factors. The delay in reactivation and the effect of anti-NGF antibody largely disappeared at 36 h after excision of TG.

Reactivation of HSV-1 from TG. HSV-1 reactivated from TG incubated after excision in medium containing NGF and EGF is accelerated by the proapoptotic drugs dexamethasone and DCPE. Concurrently, the amounts of LAT and miRNAs decreased. TG harvested 30 d after corneal infection were incubated in medium containing anti-NGF antibody (Figs. 2 and 3, columns 2 and 7), medium containing NGF+EGF (columns 3 and 8), or NGF+EGF and either dexamethasone (50 or 10 μ M) (Fig. 2, columns 4, 5, 9, 10) or DCPE (100 or 50 mM) (Fig. 3, columns 4, 5, 9, 10). All TG were individually extracted and assayed for the amount of mRNAs encoding ICP27 (α), TK (β), VP16 (γ_1), or U_L41 (γ_2) mRNAs as well as LAT and miR-H3, -H5, and -H6. Included in these assays were individual ganglia processed immediately after excision (Figs. 2 and 3, columns 1 and 6). The results were as follows:

- The amounts of viral mRNAs increased ~100-fold between the time of excision and the 24 h time point (Figs. 2 and 3, columns 1 and 2). At the same time, as previously reported, the amounts of LAT, miR-H3, miR-H5, and miR-H6 decreased at least 10-fold.
- NGF and EGF depressed the accumulation of viral mRNAs during the same time interval. The increase was less than

Fig. 1. Incubation of TG in medium containing NGF+EGF delays accumulation of viral mRNAs. TG excised from mice 30 d after corneal inoculation were incubated in medium 199V containing anti-NGF antibody, NGF, or NGF+EGF (300 ng/mL each). The TG were individually processed at times shown and as described in *Materials and Methods*. The results shown are the geometric mean per groups of six ganglia selected at random.

Fig. 2. Dexamethasone accelerates the reactivation of latent HSV from TG maintained in medium containing NGF+EGF. TG were excised from mice 30 d after corneal inoculation and incubated in medium 199V containing anti-NGF antibody, NGF+EGF, or NGF+EGF and dexamethasone (50 or 10 μ M). At times shown individual ganglia were processed as described in *Materials and Methods*. The figure shows the geometric mean amounts of viral mRNAs or viral miRNAs and LATs normalized with respect to cellular RNA.

- fivefold. In contrast, LAT and the miRNAs decreased two- to (at most) threefold (Figs. 2 and 3, columns 3 and 8).
- Both doses of dexamethasone in medium containing NGF and EGF caused an increase in the accumulation of viral mRNAs and a decrease in LAT and miRNA to levels seen in TG incubated in medium containing anti-NGF antibody (compare Fig. 2, columns 4 and 5, with column 2, and columns 9 and 10 with column 7).
- Only the higher dose of DCPE had an effect on activation of HSV-1 and repression of accumulation of LAT and miRNAs similar to those observed in TG incubated in the presence of dexamethasone.

We conclude that dexamethasone or DCPE accelerate the activation of viral gene expression in TG excised and incubated in medium containing NGF and EGF.

Viral DNA Accumulates in TG Excised and Maintained in Organ Culture in the Presence and Absence of Dexamethasone. TG ganglia excised 30 d after corneal inoculation were harvested and DNA was extracted immediately or at intervals after excision and incubation in medium containing anti-NGF antibody (Fig. 4A). In this experiment, viral DNA increased 10-fold from the time of excision of TG (time 0) and 24 h of incubation. The amount detected is the maximum that could be detected during the single cycle of reactivation in the absence of the drug. In dexamethasone-treated TG (50 μ M, Fig. 4B) incubated in medium containing NGF+EGF, the amount of viral DNA increased a mere threefold in cells.

We conclude the following:

Fig. 3. The highest concentration of DCPE tested accelerates the reactivation of latent HSV from TG maintained in medium containing NGF+EGF. TG were excised from mice 30 d after corneal inoculation and incubated in medium 199V containing anti-NGF antibody, NGF+EGF, or NGF+EGF and DCPE (50 or 100 nM). At times shown individual ganglia were processed as described in *Materials and Methods*. The figure shows the geometric mean amounts of viral mRNAs or viral miRNAs and LATs normalized with respect to cellular RNA.

- The synthesis of viral DNA indicates that the activated neurons synthesize viral proteins required for viral DNA synthesis.
- The amount shown assumes that viral DNA synthesis takes place in all neurons harboring latent virus. If this is not the case, the actual amount of viral DNA synthesis in neurons in which viral reactivation took place was much higher.
- Although dexamethasone induced the synthesis and accumulation of viral mRNAs to a level similar to that seen in TG incubated in medium containing anti-NGF antibodies, the amount of viral DNA made in drug-induced TG was lower.

Dexamethasone-Induced Apoptosis in TG Ganglia Incubated After Excision in Medium Containing the Drug. Two series of experiments were done to determine whether, at the dose used, dexamethasone induced apoptosis in TG and particularly in neurons. In the first series of experiments, TG were excised, incubated in medium with and without dexamethasone (50 μ M) for 16 h and then solubilized, subjected to electrophoresis in denaturing gels, transferred to a nitrocellulose sheet, and reacted with antibody to PARP or cleaved caspase 3. As shown in Fig. 4C, both cleaved poly (ADP-ribose) polymerase (PARP) and caspase 3 were readily demonstrable by immunoblotting with respective antibodies. In the second experiment, the dexamethasone treated and untreated ganglia as above were fixed, sectioned, and reacted with antibody to cleaved caspase 3. The arrows in Fig. 4D point to neurons stained with the antibody to cleaved caspase 3. These neurons appear shriveled and smaller in size in contrast to the neurons seen in sections of ganglia incubated in medium in the absence of the drug (control panel).

Dexamethasone Accelerates the Expression of Viral Genes and the Accumulation of Infectious Virus in Cultured Cells. Two series of experiments were done. In the first series of experiments, replicate cultures of SK-N-SH or Hep-2 cells were exposed to dexamethasone (50 μ M) 4 h before exposure of cells to 0.5 pfu of HSV-1(F) per cell. At intervals shown in Fig. 5A, a replicate culture was harvested and processed as described in *Materials and Methods*, and representative viral mRNAs were quantified by RT-qPCR. The results shown in Fig. 5B and C were as follows:

- The amounts of mRNAs detected at 3, 5, and 8 h after infection were normalized with respect to the amounts detected at 1 h after exposure of virus to cells. Because α -mRNAs (ICP0, ICP27) are already present in sizable amounts at 1 h after infection, the relative increase in the amounts of these mRNAs at 3, 5, or 8 h after infection was not as great as that of β - or γ -mRNAs at later time points.
- In SK-N-SH cells, dexamethasone accelerated (e.g., ICP0) or induced higher levels of expression of viral mRNAs (e.g., ICP0, ICP27, TK, VP16) than the untreated infected cells. In Hep-2 cells, we noted acceleration of expression of α -mRNAs (ICP0 and ICP27) but not that of VP16 mRNA.

The second series of experiments was similar to the first except that the cultures were harvested at 1, 3, 5, 8, and 24 h after

Fig. 4. Studies on viral DNA synthesis and induction of apoptosis in untreated and dexamethasone treated TG. (A and B) Quantification of viral DNA copies in TG. TG ganglia excised from 30 d after corneal-inoculated mice were incubated in medium 199v containing anti-NGF antibody (A) or medium containing NGF+EGF+dexamethasone (50 μ M) (B). The number of viral DNA copies per 50 ng of DNA from TG was determined by qPCR and are shown as geometric mean titers based on assays of individual ganglia. (C and D) Induction of apoptosis in TG following incubation in medium containing dexamethasone. TG were harvested 16 h after excision and processed as described in *Materials and Methods*. (D) TG were incubated as above but were fixed, sectioned, and reacted with antibody to cleaved caspase 3 as detailed in *Materials and Methods*.

This requirement is met by the TG organ cultures described in our studies, and we have also noted that, in this system, reactivation recapitulates what actually happens in vivo because, as noted in the Introduction, neurons and satellite cells communicate and regulate each other. The alternative model (e.g., 34) involves studies on neurons isolated from ganglia and “cured” by maintenance for several days in acyclovir, a drug that induces chain termination and single-stranded gaps in both cellular and viral DNAs in cells infected with HSV and expressing viral thymidine kinase (reviewed in ref. 1). One consequence of relying on acyclovir for viral gene suppression is the potentially aberrant transcription due to the single-stranded gaps similar to those in replicating DNA that favor late gene transcription and the necessity for viral DNA repair before viral replication can resume. The data obtained on isolated neurons in studies published to date do not conform to the requirement that reactivation take place within the time frame of a single cycle of viral replication from the time of excision of the ganglia harboring the latent virus.

Materials and Methods

Cells and Virus. Vero, SK-N-SH, and HEp-2 cell lines were purchased from ATCC. HSV-1(F) is a limited passage prototype strain used in this laboratory (35). The cells and TG excised from mice were maintained in mixture 199 supplemented with 1% calf serum (199V) (2).

Reagents. Monoclonal antibodies to PARP and β -actin were purchased from Santa Cruz Biotechnology and Sigma, respectively. Cleaved Caspase-3 (As175), rabbit mAb (#9664 for IHC), and rabbit polyclonal antibody (#9661 for WB) were purchased from Cell Signaling Technology, Inc. Dexamethasone and DCPE were purchased from Sigma and ChemBridge.

Real-Time PCR Analyses. Total RNA extraction from SK-N-SH and HEp-2 cells, cDNA synthesis and RT-PCR conduction by using primers for ICP0, ICP27, TK, and VP16 were reported elsewhere (36). Quantification of viral DNA copy

numbers in TG was performed by SYBR green real-time PCR technology (StepOnePlus System, ABI) as reported (37).

Murine Model of Ocular Infection. Four-week-old inbred female CBA/J mice (Jackson Labs) receiving unrestricted access to food and water were infected by corneal route as reported previously (2). All animal studies were done according to protocols approved by the University of Chicago Animal Care Committee.

Induction of Apoptosis and Virus Reactivation. TG were removed 30 d after infection and incubation at 37 °C, plus 5% CO₂ in medium 199V alone or with NGF and EGF (300 ng each per mL) in presence or absence of the proapoptotic drugs. Reactivation of latently infected virus from TG was reported elsewhere (2).

Immunohistochemistry. TG were excised and incubated in 199v medium containing dexamethasone (50 μ M) at 37 °C for 16 h and then were fixed with 10% neutral formalin for 6 h at room temperature and sliced into 10 μ m in thickness and stored at –80 °C. For staining, the sections were brought to room temperature, rinsed with PBS, reacted with antigen retrieval buffer (DAKO, S1699), and heated in a steamer for 20 min at 97 °C. The slides were reacted with 3% H₂O₂ for 5 min and then reacted with anti-cleaved caspase 3 rabbit mAb (#9664, Cell Signaling). Bound antibody was detected by the Envision+ anti-rabbit system (K4002, DAKO). The slides were briefly immersed in hematoxylin for counterstaining and evaluated under light microscope.

Immunoblot of TG Tissues. TG were excised and incubated in medium 199V containing dexamethasone (50 μ M) at 37 °C for 16 h. The TGs were homogenized and sonicated in 150 μ L of protein lysis buffer (36). After addition of 150 μ L of 2 $\times$ final concentration of SDS loading buffer, 150 μ L of samples were electrophoretically separated in a 10% PAGE gel and reacted with the appropriate primary antibodies.

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