Effect of Protein Kinase C Inhibitors on the Antiviral Activity of Human Alpha Interferon in Herpes Simplex Virus-Infected Human Neuroblastoma Cells

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Pretreatment of human neuroblastoma cells with an inhibitor of protein kinase C (PKC), staurosporine or H-7, prior to the addition of human alpha interferon (HuIFN- α), recombinant HuIFN- α , or recombinant HuIFN- β blocked the inhibitory effect of these IFNs on the release of infectious herpes simplex virus type 1 from treated cells. In addition, staurosporine blocked the inhibitory effect of HuIFNs on the expressions of herpes simplex type 1 glycoproteins B, C, and D in treated neuroblastoma cells. Furthermore, addition of HuIFNs resulted in an increased expression of PKC in treated neuroblastoma cells. These results suggest that inhibitors of PKC block the expression of HuIFN-induced genes in treated human neuroblastoma cells. Thus, the activation of PKC is an important step in the HuIFN-treated cells of neuronal origin.

It is known that herpes simplex virus (HSV) infection, in addition to leading to symptomatic primary disease, may lead to the development of recurrent lesions following reactivation of the latent virus in the neurons of sensory ganglia (1, 22, 23). In recent years, significant efforts have been made to inhibit recurrent and primary HSV infections by use of a variety of antiviral agents. Reports from our and other laboratories have shown that human interferons (HuIFNs) can block HSV replication in different cell lines, including cells of neuronal origin, a site for establishment of latency (2–4, 7, 11, 15, 18). In addition, we reported that HuIFN- α differentially affected the expression of HSV type 1 (HSV-1) glycoprotein B (gB), gD, and gE in human neuroblastoma cells (2). However, the exact mechanism of action of HuIFN against HSV-1 replication and especially glycoprotein expression in neuroblastoma cells is unknown. Recently Pfeffer et al. (20) demonstrated that IFN- α inhibited the replication of vesicular stomatitis virus by a selective activation of protein kinase C (PKC). This enzyme plays a central role in the signal transduction pathway and has been implicated in the regulation of a variety of cellular processes, including cell differentiation and release of growth hormones and neurotransmitters (17). Molecular cloning has demonstrated that PKC consists of at least eight isoforms (17, 19).

In this communication, we report that selective inhibitors of PKC, staurosporine and H-7, blocked the antiviral effect of HuIFNs on the replication of HSV-1 in human neuroblastoma cells. Pretreatment of cells with staurosporine also reduced the inhibitory effect of HuIFNs on the expression of HSV-1 gB, gC, and gD. Furthermore, using an antipeptide antibody against PKC, we demonstrated that the addition of HuIFN- α induced a protein identical in molecular weight to PKC in human neuroblastoma cells.

To determine the effect of staurosporine on the antiviral activity of HuIFNs, human neuroblastoma cells were pretreated with 0.01 μ g of staurosporine per ml for 1 h and then treated with 100 U of HuIFN- α , recombinant HuIFN- α (rHuIFN- α), or rHuIFN- β per ml for 18 h. The concentration

of staurosporine was selected from a dose-response experiment in order to determine the optimum concentration. One set of cells served as an untreated control. Cells were then infected with strain F of HSV-1 (provided by B. Roizman, University of Chicago, Chicago, Ill.) at a multiplicity of infection of 5. Supernatant fluids from the treated and untreated cells were collected 48 h postinfection and tested for the ability to form plaques on African green monkey kidney (BS-C-1) cells. The plaques were counted by light microscopy after the cells were stained with May-Grunwald and Giemsa stains (16). The result of this experiment (Table 1) reveals that the addition of staurosporine almost completely blocked the antiviral effect of all HuIFNs tested (HuIFN- α , rHuIFN- α , and rHuIFN- β) in human neuroblastoma cells. However, it is interesting that staurosporine had no significant effect on the antiviral activity of HuIFN in nonneuronal cells, such as human fibroblast cells (Table 1).

To confirm the results obtained after staurosporine treatment, we treated human neuroblastoma cells with another inhibitor of PKC, H-7 (20). It was noticed that addition of H-7 also blocked the antiviral effect of HuIFN- α in human neuroblastoma cells (Table 2). In parallel, we used HA-1004, a structurally similar but weak inhibitor of PKC (20), as a control. The result of this experiment revealed that HA-1004 had no significant effect on the antiviral activity of HuIFN- α in human neuroblastoma cells (Table 2).

Previous studies from this laboratory showed that HuIFN- α differentially affected the expression of HSV-1 glycoproteins in human neuroblastoma cells (2). It was therefore of interest to determine whether staurosporine could reverse the effect of HuIFNs on the expression of HSV-1 glycoproteins in neuroblastoma cells. In brief, cell lysates were collected from the previous experiment and processed for polyacrylamide gel electrophoresis and subsequent immunoblotting as previously described (3, 24). The resulting blots were incubated with monoclonal antibodies to gB, gC, and gD developed in this laboratory (12). Nitrocellulose strips were further incubated with rabbit anti-mouse immunoglobulin G (Organon Teknica Corporation, West Chester, Pa.). Finally, the nitrocellulose strips were treated with [¹²⁵I]protein A for 1 h and processed for autoradiography. Figure 1 displays the result of this exper-

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TABLE 1. Effects of staurosporine on the antiviral activities of HuIFNs^a

Cells	Addition	No. of PFU/ml	% Inhibition
Human neuroblastoma	0	5.0×10^5	0
	HuIFN- α (100 U/ml)	3.0×10^4	94
	Staurosporine (0.01 μ g/ml) + HuIFN- α (100 U/ml)	6.1×10^5	0
	0	1.4×10^5	0
	rHuIFN- α (100 U/ml)	1.0×10^4	93
	Staurosporine (0.01 μ g/ml) + rHuIFN- α (100 U/ml)	2.2×10^5	0
	0	4.1×10^5	0
	rHuIFN- β (100 U/ml)	1.1×10^4	97.3
	Staurosporine (0.01 μ g/ml) + rHuIFN- β (100 U/ml)	4.0×10^5	2.5
Human fibroblast	0	3.9×10^5	0
	HuIFN- α (100 U/ml)	8.2×10^3	97.9
	Staurosporine (0.01 μ g/ml) + HuIFN- α (100 U/ml)	7.0×10^3	98.2

^a Human neuroblastoma and human fibroblast cells were pretreated with staurosporine (obtained from Upstate Biotechnology, Inc., Lake Placid, N.Y.) for 1 h, washed, and then treated with different HuIFNs for 18 h. HuIFN- α , rHuIFN- α , and rHuIFN- β were obtained from JCR Pharmaceuticals Co. (Kobe, Japan), Schering-Plough Research (Bloomfield, N.J.), and Triton Biosciences, Inc. (Alameda, Calif.), respectively. One set of cells served as an untreated control. The human neuroblastoma cells were provided by T. Howard, University of Alabama at Birmingham. These cells exhibited rosette formation, had microtubule arrays and neurofilaments, and exhibited neuron-specific enolase activity. Cells were then infected with strain F of HSV-1. Supernatants from treated and untreated cells were tested for the ability to form plaques as described in the text.

iment. The levels of gB and gC were greatly reduced in IFN-treated neuroblastoma cells. On the other hand, gD, although reduced in quantity, was expressed after IFN treatment as previously reported (2). Addition of staurosporine, however, significantly blocked the inhibitory effect of HuIFN- α and rHuIFN- β on the expression of these glycoproteins in human neuroblastoma cells (Fig. 1). Staurosporine also reversed the effect of HuIFNs on the expression of glycoproteins in the total extracellular viral particles. We previously showed that the extracellular particles released from HuIFN- α -treated neuroblastoma cells expressed very little of the viral glycoproteins tested (2). In brief, human neuroblastoma cells were pretreated with staurosporine and HuIFN- α as previously described. Cells were then infected with HSV-1. The culture supernatants were collected and clarified, and the virus was pelleted by centrifugation at 40,000 rpm for 1 h. The pellets were lysed and subjected to polyacrylamide gel electrophoresis and subsequent immunoblotting. The nitrocellulose blots were reacted with monoclonal antibodies to gB, gC, and gD. Figure 2 demonstrates that the pretreatment of neuroblastoma cells with staurosporine almost completely blocked the inhibitory effect of HuIFN- α on the expression of gB, gC, and gD in the total extracellular HSV-1 preparations released from treated cells. A similar result was obtained when rHuIFN- β was used instead of HuIFN- α (data not shown).

To determine the expression of PKC, if any, in human neuroblastoma cells after the addition of HuIFNs, we performed

the following experiment. In brief, human neuroblastoma cells were treated with different concentrations of HuIFN- α or rHuIFN- β for 18 h. One set of cells served as an untreated control. Cell lysates were collected and processed for immunoblotting as described above. The resulting blot was incubated with rabbit antipeptide antibody against PKC α (Gibco BRL, Gaithersburg, Md.). A protein band of approximately 80 kDa, corresponding to the reported molecular mass of PKC, was detected in the HuIFN-treated neuroblastoma cells (Fig. 3A). As a control, we also processed cell lysates collected from HuIFN- α -treated human fibroblast cells for immunoblotting; no protein band was detected when the blot was incubated with the same antibody to PKC (Fig. 3B).

The exact mechanism of action of HuIFNs on HSV-1, especially in cells of neuronal origin, is unknown. Previously we demonstrated that HuIFN- α significantly inhibited the replication of HSV-1 in human neuroblastoma cells (2). Furthermore, HuIFN- α differentially affected the expression of HSV-1 gB, gD, and gE in neuroblastoma cells. Recent reports from different laboratories also demonstrated that HuIFN differentially affected the expression of viral proteins in human papillomavirus- and Epstein-Barr virus-infected human cells (10, 14). Although the exact mode of action of HuIFNs on the expression of HSV-1 glycoproteins is unknown, it is apparently different from that observed after treatment of cells with tunicamycin or brefeldin A (5, 6, 8).

The results presented here revealed that staurosporine al-

TABLE 2. Effects of H-7 and HA-1004 on the antiviral activities of HuIFNs in human neuroblastoma cells^a

Inhibitor	Addition	No. of PFU/ml	% Inhibition
H-7	0	2.1×10^4	0
	HuIFN- α (100 U/ml)	2.0×10^3	90.5
	H-7 (20 μ M) + HuIFN- α (100 U/ml)	1.7×10^4	19
HA-1004	0	3.0×10^4	0
	HuIFN- α (100 U/ml)	4.0×10^3	86.7
	HA-1004 (20 μ M) + HuIFN- α (100 U/ml)	5.0×10^3	83.3

^a The experimental procedure was essentially same as described in the footnote to Table 1. H-7 and HA-1004 were purchased from Sigma Chemical Co., St. Louis, Mo. The concentrations were selected from a dose-response experiment to determine the optimum concentration.

FIG. 1. Effects of staurosporine on the antiviral activity of HuIFNs and on the expression of HSV-1 gB, gC, and gD in human neuroblastoma cells. (A and C) Nitrocellulose blots were incubated with monoclonal antibodies to gB and gD. (B and D) Nitrocellulose blots were incubated with monoclonal antibodies to gC. (A and B) Cell lysates from HuIFN- α -treated neuroblastoma cells. (C and D) Cell lysates from rHuIFN- β -treated neuroblastoma cells. Lane 1, no IFN; lane 2, 100 U of IFN per ml; lane 3, 0.01 μ g of staurosporine per ml plus 100 U of IFN per ml.

most completely reversed the antiviral effect of all the HuIFNs tested in human neuroblastoma cells, probably by preventing the activation of HuIFN-induced genes (20). Studies from other laboratories have suggested that translation of viral mRNA can be inhibited in several ways in IFN-treated cells (13). Two major effects on translation have been observed: (i) through a protein kinase that blocks translation of certain viral proteins by phosphorylation of the initiation factor eIF-2, and (ii) through a specific 2,5-oligoadenylate-activated endonuclease (9, 21, 25). Recently Pfeffer et al. (20) reported that IFN- α blocked the replication of vesicular stomatitis virus by a selective induction of PKC, an enzyme which plays an important role in the signal transduction pathway. Our results also indicated that the expression of PKC α was an important step in HuIFN-treated neuroblastoma cells. At present, the possibility of involvement of other enzyme systems in addition to PKC α in HuIFN-treated human neuroblastoma cells cannot be ruled out. Future investigations aimed at addressing this issue and other possibilities should help in understanding in more detail

FIG. 2. Expression of extracellular viral glycoproteins after HuIFN- α treatment. The experimental procedure is described in the text. (A) Nitrocellulose blot reacted with monoclonal antibodies to gB and gD. (B) Blot reacted with monoclonal antibodies to gC. Lane 1, no IFN; lane 2, 200 U of IFN per ml; lane 3, 0.01 μ g of staurosporine per ml plus 200 U of IFN per ml.

FIG. 3. Expression of PKC in HuIFN-treated human neuroblastoma and human fibroblast cells. The experimental procedure is described in the text. (A) Human neuroblastoma cells. Lane 1, no IFN; lane 2, 100 U of HuIFN- α per ml; lane 3, 200 U of HuIFN- α per ml; lane 4, 10 U of rHuIFN- β per ml; lane 5, 100 U of rHuIFN- β per ml. Expression of the 80-kDa (80K) protein is indicated by an arrow. (B) Human fibroblast cells. Lane 1, no IFN; lane 2, 100 U of HuIFN- α per ml; lane 3, 200 U of HuIFN- α per ml.

the mechanism of action of HuIFNs, especially in cells of neuronal origin, a site for establishment of latency.

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