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### **Research Article**

Intrauterine growth retardation and neurodevelopmental handicaps are common among infants born to HIV-positive mothers and may be due to the actions of virions and/or maternally derived viral products. The viral envelope protein, gp120, is toxic to neurons, induces neuronal dystrophy, and retards behavioral development in neonatal rats. Vasoactive intestinal peptide, a neuropeptide regulator of early postimplantation embryonic growth, and the neuroprotective protein, activity-dependent neurotrophic factor, prevent gp120-induced neurotoxicity. Whole embryo culture of gestational day 9.5 mouse embryos was used to assess the effect of gp120 on growth. Embryos treated with gp120 exhibited a dose-dependent inhibition of growth. gp120-treated embryos (10(-8) M) grew 1.2 somites in the 6-h incubation period, compared with 3.9 somites by control embryos. Embryos treated with gp120 were significantly smaller in cross-sectional area and had significantly less DNA and protein than controls. Growth inhibition induced by gp120 was prevented by cotreatment with vasoactive intestinal peptide or activity-dependent neurotrophic factor. gp120 may play a role in the growth retardation and developmental delays experienced by infants born to HIV-positive mothers. Vasoactive intestinal peptide and related factors may provide a therapeutic strategy in preventing developmental deficits.

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## Inhibition of Murine Embryonic Growth by Human Immunodeficiency Virus Envelope Protein and Its Prevention by Vasoactive Intestinal Peptide and Activity-dependent Neurotrophic Factor

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### Abstract

Intrauterine growth retardation and neurodevelopmental handicaps are common among infants born to HIV-positive mothers and may be due to the actions of virions and/or maternally derived viral products. The viral envelope protein, gp120, is toxic to neurons, induces neuronal dystrophy, and retards behavioral development in neonatal rats. Vasoactive intestinal peptide, a neuropeptide regulator of early post-implantation embryonic growth, and the neuroprotective protein, activity-dependent neurotrophic factor, prevent gp120-induced neurotoxicity. Whole embryo culture of gestational day 9.5 mouse embryos was used to assess the effect of gp120 on growth. Embryos treated with gp120 exhibited a dose-dependent inhibition of growth. gp120-treated embryos ( $10^{-8}$  M) grew 1.2 somites in the 6-h incubation period, compared with 3.9 somites by control embryos. Embryos treated with gp120 were significantly smaller in cross-sectional area and had significantly less DNA and protein than controls. Growth inhibition induced by gp120 was prevented by cotreatment with vasoactive intestinal peptide or activity-dependent neurotrophic factor. gp120 may play a role in the growth retardation and developmental delays experienced by infants born to HIV-positive mothers. Vasoactive intestinal peptide and related factors may provide a therapeutic strategy in preventing developmental deficits. (*J. Clin. Invest.* 1997. 99:2837–2841.) Key words: vasoactive intestinal peptide • activity-dependent neurotrophic factor • embryo • human immunodeficiency virus gp120 • growth retardation

### Introduction

Low birth weight as a result of intrauterine growth retardation is associated with high perinatal mortality, morbidity, and neurodevelopmental disorders (1) and is a common feature among the infants of HIV-infected mothers (2–14). Emerging

evidence indicates that both infected and uninfected infants of HIV-positive mothers can be developmentally compromised, suggesting a direct effect of maternal HIV infection (9–15). In several studies, either from populations in which drug use is not common or excluding data from mothers with known drug use, both HIV-infected and uninfected infants of HIV-positive mothers weighed significantly less at birth than the infants of uninfected mothers (9–14). In addition, a recent proton magnetic resonance spectroscopy study demonstrated central nervous system abnormalities in all infants born to HIV-infected mothers compared with control infants (15). The HIV envelope protein, gp120, has toxic properties (16–30) and can form toxic fragments in vivo (17). This viral protein, therefore, is a leading candidate as an injurious agent to both mother and infant during pregnancy, regardless of the infectious state of the infant.

The resistance of rodents to infection by HIV, coupled with their vulnerability to the toxicity of gp120, has provided a way to assess the contribution of gp120 to the pathology of HIV infection. gp120 was first shown to be potentially toxic to hippocampal neurons in culture (16), and subsequent work reported similar effects in retinal ganglion cells (18, 19). Rats exposed to gp120 exhibited extensive neuronal pathology, behavioral retardation (17), and learning impairment (20), which appeared to be due, at least in part, to the in vivo generation of low molecular weight toxic fragments of gp120 (17). Neuronal dysmorphism and cell death were also evident in transgenic mice expressing gp120 (21). gp120 has also been shown to contribute to immunoregulatory dysfunction by interfering with the activity of cells of the immune system (22–30).

The neurotoxicity exhibited by gp120 in mixed central nervous system (CNS)<sup>1</sup> cultures can be prevented by vasoactive intestinal peptide (VIP) (16), a CNS neurotransmitter with neurotrophic properties (31, 32). VIP is a regulator of early postimplantation growth during embryonic days (E) 9–11 in the mouse, acting through receptors localized to the CNS (33–35). This period of development follows neural tube closure and the beginning of neurogenesis, and is characterized by critical developmental events, including embryogenesis, and the initiation of placental nutrition. With whole embryo culture, a technique allowing precision in developmental timing and

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1. Abbreviations used in this paper: ADNF, activity-dependent neurotrophic factor; CNS, central nervous system; E, embryonic day; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide.

drug delivery, VIP caused a dramatic increase in the growth of E9.5 mouse embryos (33). In addition, treatment of pregnant mice during the early postimplantation period (E9–11) with a VIP antagonist not only inhibited total embryonic growth, but also produced microcephaly by inducing a disproportionately greater inhibition of brain growth compared with body growth (34).

Many actions of VIP are indirect, occurring through the VIP-stimulated release of several factors, including the novel neuroprotective factor, activity-dependent neurotrophic factor (ADNF) (36–38). ADNF may account for many of the neurotrophic properties of VIP since it is released from glial cells upon stimulation by VIP (38). ADNF has a sequence homology with heat shock protein 60, and also has growth-promoting action on early postimplantation mouse embryos (39). At femtomolar concentrations, ADNF and ADNF-related peptides have been shown to prevent the neurotoxicity associated with gp120, beta-amyloid protein, and electrical blockade in CNS cultures (38).

The purpose of the present study was to examine the effects of gp120 on the growth of whole cultured early postimplantation E9.5 mouse embryos. This embryonic stage is comparable with ~ 3.5-wk gestation in the human (40), a highly susceptible period for teratogenic events. Additionally, the protective actions of VIP and ADNF were evaluated. Growth was assessed by measurement of a number of somites (a growth indicator; reference 41), cross-sectional area, and total DNA and protein content.

## Methods

National Institutes of Health (NIH) Swiss mouse embryos were explanted at E9.5. Embryos 14–19 somites in size, with yolk sac and ectoplacental cone intact, were selected and cultured in adult male Sprague-Dawley rat serum supplied by Harlan Sprague Dawley Inc. (Indianapolis, IN) for 6 h at 37°C, in a gas phase of 5% CO<sub>2</sub>/95% air as previously described (33, 42, 43). Pairs of embryos were cultured in 1.0 ml serum, in polypropylene tubes placed on a rotating agitator (30 rpm). Recombinant gp120/HIV-1SF2 from the AIDS Reagent Program, Ogden Bioservices Corp. (Rockville, MD) was added to the serum at concentrations varying from  $3 \times 10^{-13}$  to  $3 \times 10^{-8}$  M. VIP obtained from Peninsula Laboratories, Inc. (Belmont, CA) ( $10^{-7}$  M) and ADNF, extracted and purified as previously described (38) ( $10^{-13}$  M) were added, both alone and as a cotreatment with gp120 at  $3 \times 10^{-9}$  M. Additional controls using recombinant baculovirus-expressed gp160 from Repligen Corp. (Cambridge, MA) at  $3 \times 10^{-9}$  M, secretin (from rat; Peninsula Laboratories, Inc.) at  $10^{-7}$  M and pituitary adenylate cyclase-activating polypeptide (PACAP) 38 (a generous gift from Dr. Akira Arimura, Tulane University, Hebert Ctr., Belle Chasse, LA) at  $10^{-13}$ ,  $10^{-11}$ ,  $10^{-9}$ , and  $10^{-7}$  M were also performed. All solutions were made up in PBS, except VIP and PACAP, which were diluted with 0.02% acetic acid. The culture medium was not modified during the culture period. After 6 h, the number of somites was counted and measurement of the total cross-sectional area of the body was made using an image analyzer (IMAGE; Wayne Rasband, Research Services Branch, National Institute of Mental Health). Embryos were frozen on dry ice and stored at  $-70^{\circ}\text{C}$  for DNA and protein content analysis. For DNA and protein analysis, frozen embryos were thawed and homogenized in PBS. Total DNA was quantitated using the method of Burton (44), modified by Munro (45), and total protein assayed by the method of Bradford (46), using the Protein Assay Reagent (Bio-Rad Laboratories, Melville, NY). Data were analyzed by ANOVA using StatView software by Abacus Concepts, Inc. (Berkeley, CA), followed by comparisons between controls and all other treatment groups.

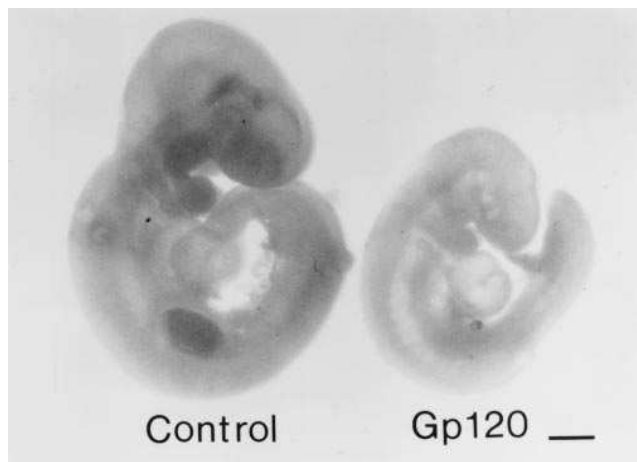


Figure 1. Photomicrograph of E9 mouse embryos after 6-h whole embryo culture in rat serum. Initial somite count for both embryos was 18. Left, control embryo; right, embryo treated with gp120 at  $3 \times 10^{-8}$  M. Sagittal view, bar = 0.5 mm.

## Results

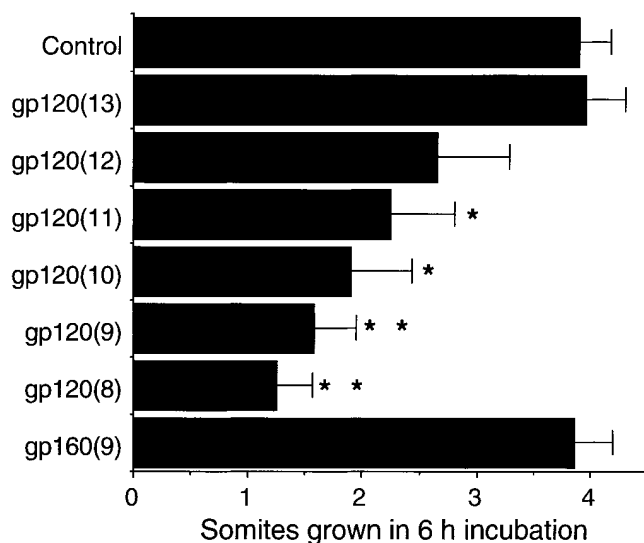
gp120 inhibited the growth of early postimplantation embryos (Fig. 1, Table I) without causing apparent malformations (Fig. 1). Whole cultured E9.5 mouse embryos treated with gp120 at  $3 \times 10^{-8}$  M grew only one third as many somites as control embryos during the 6-h incubation period (Fig. 2). Embryos treated with  $3 \times 10^{-9}$  M gp120 were 14% smaller in cross-sectional area and contained 40% less DNA and 45% less protein than controls (Table I). The growth retardation exhibited a dose-dependent response over a  $10^5$ -fold range of gp120 concentrations, with the greatest effect evident using  $3 \times 10^{-8}$  M gp120; however, significant inhibition of somite growth was seen at all concentrations  $> 3 \times 10^{-12}$  M (Fig. 2). Recombinant baculovirus-expressed gp160, the HIV protein precursor of gp120 and gp41, did not inhibit embryonic growth (Fig. 2), which is consistent with previous studies using cultured neurons in which gp120, but not recombinant gp160, was shown to be neurotoxic (16).

Cotreatment with gp120 and either VIP or ADNF resulted in control levels of somite growth, total body cross-sectional area, and DNA and protein content (Fig. 3, Table I). A 6-h treatment with  $10^{-7}$  M VIP alone resulted in growth significantly greater than control embryos (Fig. 3). In previous stud-

Table I. Measurements of Embryonic Growth

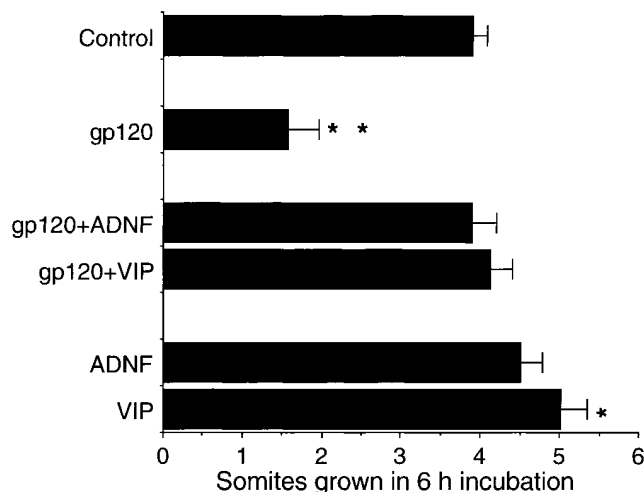
Treatment	Size	Protein	DNA
	mm <sup>2</sup>	mg	ng
Control	4.94±0.12	0.18±0.02	11.42±1.24
gp120	4.29±0.10*	0.10±0.01‡	6.85±0.92§
gp120 + VIP	4.91±0.14	0.17±0.02	10.23±0.96
gp120 + ADNF	4.87±0.20	0.16±0.01	10.63±1.18

gp120 ( $3 \times 10^{-9}$  M) inhibits embryonic growth. Cotreatment with VIP and ADNF prevents gp120-induced growth inhibition. Results are given as mean±SEM. A minimum of eight embryos were measured in each treatment group. Compared with control, \* $P < 0.001$ , † $P < 0.01$ , § $P < 0.02$ .



**Figure 2.** Mean somites grown ( $\pm$ SEM) in 6-h incubation of E9.5 mouse embryos. Treated embryos were cultured with recombinant gp120SF2 at concentrations ranging from  $3 \times 10^{-13}$  M to  $3 \times 10^{-8}$  M. A minimum of eight embryos was included in each treatment group. gp120 treatment at  $3 \times 10^{-8}$  and  $3 \times 10^{-9}$  M resulted in embryos significantly smaller than controls (\*\* $P < 0.0001$ ). gp120 at  $3 \times 10^{-10}$  and  $3 \times 10^{-11}$  M also resulted in significant growth retardation (\* $P < 0.01$ ). gp160-treated ( $3 \times 10^{-9}$  M) embryos did not differ significantly from controls.

ies, a 4-h incubation with  $10^{-13}$  M ADFN alone induced significant growth over controls (39); however, during the 6-h incubation used here, the increased growth rate did not continue to surpass that of untreated embryos ( $P < 0.07$ ) (Fig. 3). Secretin ( $10^{-7}$  M), a closely related polypeptide from the VIP



**Figure 3.** Mean somites grown ( $\pm$ SEM) in 6-h incubation of E9.5 mouse embryos. Treated embryos were cultured with recombinant gp120SF2 at  $3 \times 10^{-9}$  M. A minimum of 22 embryos were included in each treatment group. gp120 treatment at  $3 \times 10^{-9}$  M resulted in embryos significantly smaller than controls (\*\* $P < 0.0001$ ). Cotreatment with VIP ( $10^{-7}$  M) or ADFN ( $3 \times 10^{-13}$  M) resulted in embryos that did not differ from controls. VIP treatment alone ( $10^{-7}$  M) resulted in embryos larger than controls (\* $P < 0.001$ ).

family, neither induced growth in cultured embryos nor prevented gp120-induced growth retardation (data not shown). PACAP 38, which also belongs to the VIP family of peptides, had no effect on embryonic growth at the concentrations of  $10^{-13}$ ,  $10^{-11}$ , or  $10^{-9}$  M. However,  $10^{-7}$  M PACAP had the opposite effect of VIP at this concentration and significantly inhibited the growth of embryos.

## Discussion

This study demonstrates that extremely low concentrations of the HIV viral envelope protein, gp120, can inhibit embryonic growth and suggests that the growth retardation reported in both infected and uninfected infants of HIV-positive mothers may be due to maternally derived gp120 or in vivo-generated toxic gp120 fragments interfering with the normal growth-regulatory mechanisms of the developing embryo/fetus. These data also suggest that the potential growth-retarding effects of gp120 will vary depending on the concentration of gp120 and the stage in development in which exposure occurs. gp120 has been measured at concentrations from  $1.0$ – $7.6 \times 10^{-10}$  M in the serum of AIDS patients (47), and potent neurotoxicity has been observed in the cerebrospinal fluid of HIV-infected patients (48). The above concentrations are within the range at which we show a significant effect on embryonic growth. We have recently shown in a rodent model that gp120 can cross the placental barrier from maternal blood to fetal tissues (49). In addition, others have shown that, as early as the first trimester of human gestation, HIV antigens have been demonstrated in amniotic fluid and placental and fetal tissue (50, 51).

A recent study reported growth failure in transgenic mice expressing a nonreplicating HIV-1 genome encoding envelope and accessory gene products (52). The growth retardation was evident in neonates, but not in prenatal animals. Although HIV gene expression was abundant in the eye, skin, and skeletal muscle of 10-d-old mice, very low expression was present in the CNS. The presence of viral proteins in pre- or postnatal tissues was not indicated. This study also concluded the HIV gene expression, independent of viral replication, was sufficient to induce growth deficits and implicate virally encoded products in the pathogenesis of growth retardation.

The mechanism of gp120-induced neurotoxicity is unknown. Earlier work from our laboratory indicated that gp120 acts indirectly to interfere with glial cell neurotrophic functions (16, 17). VIP stimulates the release of trophic substances from astroglia. One of these secreted products is a novel neuroprotective protein (ADNF) that can prevent neuronal cell death associated with gp120 (38) and also has growth-promoting action on early postimplantation mouse embryos (39). The protection provided by VIP and its secreted product, ADFN, from gp120-induced toxicity suggests that gp120 blocks the trophic actions of these factors. Interestingly, amino acids 7–11 of VIP are similar to an amino acid sequence from the V2 region of gp120. It is possible that gp120, or a toxic fragment, could directly hinder the receptor binding of VIP. However, the indirect nature of gp120 neuronal toxicity, by impairment of glial function, is further supported by the demonstration that gp120 damages glial cells in human brain cell aggregates (53) and induces the glial secretion of toxic cytokines and the generation of nitric oxide (54–60). Additionally, transgenic mice with gp120 protein expressed in astrocytes exhibited both glial and neuronal damage (21).

The gp120-induced death of neurons has also been associated with a rise in intracellular calcium concentration, perhaps indirectly augmented by *N*-methyl-D-aspartate (NMDA) receptor activity, suggesting that neurons ultimately succumb to delayed excitotoxicity (18, 19, 61). In addition, the oxidative actions of nitric oxide have been implicated in the pathway of gp120-induced neuronal death (55–58, 62). A recent study has shown that VIP can also prevent a similar NMDA-induced, nitric oxide-dependent injury in lung tissue (63). Elevated levels of quinolinate (64, 65) and toxic products (including cytokines) released from macrophages/microglia have also been associated with increased levels of gp120 and may play a role in gp120 toxicity (55, 59, 60, 66, 67).

It is not known if the growth retardation elicited by gp120 involves the same pathways as gp120-induced neuronal toxicity; however, the ability of VIP and ADNF to prevent the effects of gp120 in both tissue culture and whole embryo culture suggests that the underlying mechanisms are similar. VIP stimulates embryonic growth during the early postimplantation period; inhibition of VIP during this period results in intrauterine growth retardation (33, 34). At this stage of rodent development, VIP binding sites are primarily, if not exclusively, localized to the CNS and are particularly dense along the floor plate neuroepithelium (68, 69), a region composed exclusively of glia in the rat and the center governing tissue organization during embryonic development. The high density of VIP binding sites in these regions suggests that VIP may coordinate early morphogenic events by direct action on neuroepithelial cells or indirectly through the regulation of the release of diffusible signals secreted from the floor plate. gp120-induced growth inhibition, and its prevention by VIP and ADNF, is consistent with the hypothesis that gp120 interferes with the growth-regulatory actions of VIP and/or its secreted products.

This study reports that the HIV envelope protein has growth-inhibiting actions that can be prevented by VIP or ADNF. Prenatal growth inhibition (9–13) and the similar initial growth retardation (14) seen among both infected and uninfected newborns of HIV-positive mothers could be due to the effects of exposure of the embryo/fetus to maternal gp120 during critical stages of intrauterine development. The present study also suggests a therapeutic intervention based on the apparent protective actions of VIP and ADNF. Such a strategy implemented prophylactically may provide protection from the growth retardative effects of gp120 or toxic gp120 fragments. The growth-inhibiting actions of gp120 give strong support to the concept that the developing fetus is in jeopardy not only from HIV infection, but also from HIV-related viral products.

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