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Identification of Endonuclease Activity in HIV-1 gp120 Preparations Produced Using Baculovirus Expression Systems

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ABSTRACT

Experiments undertaken with commercially available recombinantly produced human immunodeficiency virus Type 1 (HIV-1) gp120 demonstrated that the resuspended lyophilized protein, a product of the baculovirus expression system, had intrinsic nuclease activity. This nuclease activity was distinguishable from the molecular-grade bovine serum albumin that it was constituted in. The activity was thermolabile in that if the preparation was heated to 100°C for 10 min, the activity was abolished, although this did not happen when it was stored at -20°C. The nuclease activity was also Ca^{+2} - and Mg^{+2} -dependent, and had endonuclease as opposed to exonuclease activity. Zn^{+2} ions were found to inhibit the enzyme. The intensity of nuclease activity varied from batch to batch. A lyophilized homogenate of Sf9 insect cells expressing the Rho baculovirus-derived red blood cell protein 4.2 (Pallidin) was also found to have nuclease activity on reconstitution. In contrast, most, though not all E. coli-produced recombinant proteins were found to be free of nuclease activity. The use of activity gels to identify the size of the nuclease contained in the gp120 preparation was limited, because despite the use of many renaturation methods, the enzyme in the gp120 preparation could not be functionally resuscitated following sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoprecipitation studies were useful to demonstrate that nuclease activity in the gp120 preparation was functionally distinguishable from the gp120 itself. When mononuclear cells transformed with anti-CD3 were concurrently incubated with gp120 (5–40 $\mu\text{g/mL}$), internucleosomal DNA fragments characteristic of apoptosis were demonstrated in the supernatant by DNA gel electrophoresis. In the context of HIV-1 and AIDS, where the depletion of CD4^{+} T-cells has been found to be associated with apoptosis, nuclease activity intrinsic

to the gp120 preparation used in experimentation may potentially alter experimental results.

INTRODUCTION

The successful use of the insect cell/baculovirus expression strategy in the large-scale production of recombinant proteins of mammalian, insect, plant and bacterial origin has revolutionized biology, biotechnology and chemistry. This highly efficient expression system has been well characterized (6,9,11), and its ubiquitous utilization and exploitation is testament to the many factors intrinsic to the system that contribute to its popularity. These include high protein expression levels, ease and speed of genetic engineering, accommodation of large DNA inserts, post-translational protein processing (e.g., glycosylation) similar to mammalian cells and ease of insect cell growth in suspension (11).

Despite years of intensive investigation and speculation concerning the specific virological and immunological mechanisms of cell death involved in human immunodeficiency virus Type 1 (HIV-1) infection (13), certain of these still remain elusive. Nevertheless, there is mounting and conclusive evidence that the process of apoptosis is at least contributory to T-cell depletion (1,10,19,20).

Since the gp120 protein has been incontrovertibly linked to apoptotic cell death mechanisms in HIV-1 disease pathogenesis, and because there are dangers inherent in the purification of gp120 from cultures infected with the virus and from blood derived from HIV^{+} donors, gp120 is often produced in the insect/baculovirus system for subsequent use in research and commercial enterprises.

In this study, which was aimed at the evaluation of putative apoptosis-blocking strategies in cultures of peripheral blood mononuclear cells (PBMC) exposed to cross-linked gp120/anti-gp120 complexes, we noted contaminating endonuclease activity in culture supernatants containing recombinant gp120 protein that had been produced in the baculovirus expression system. While not disputing the role of apoptosis in T-

cell depletion in AIDS, this report is aimed at drawing attention to the baculovirus-associated endonuclease activity, which may confound the interpretation of studies addressing this phenomenon *in vitro*.

MATERIALS AND METHODS

Use of Special Reagents

Baculovirus-produced recombinant gp120_{IIIB} (Batch Nos. 93-545, 94-14, 94-276, 94-286 and 94-357) and IgG-purified rabbit anti-gp120 antibodies were obtained from Intracel (London, England, UK or Cambridge, MA, USA) and were reconstituted in sterile double-distilled water. Molecular biology-grade bovine serum albumin (BSA), which contained no deoxyribonucleases, ribonucleases, proteases or nicking activity (Boehringer Mannheim, Mannheim, Germany), was added to the reconstituted gp120 to give a final concentration of 1 $\mu\text{g/mL}$. To confirm that the BSA was not the source of the nuclease activity in our gp120 preparation, we incubated an aliquot of BSA with 1 μg DNA for 4 h and electrophoresed it. Visualization on an ethidium bromide-impregnated, agarose gel verified that BSA had no nuclease activity (Figure 1). Recombi-

Figure 1. The effect of 4 μg BSA on 1 μg human DNA when co-incubated at 37°C for 4 h and subsequently size-fractionated by DNA agarose gel electrophoresis. Lane 1 shows the result of DNA incubated in endonuclease buffer alone; whereas, Lane 2 shows the outcome of DNA incubated in the presence of both endonuclease buffer and BSA (4 μg).

nant gp120, Interleukin (IL)-1 α , IL-1 β , IL-6 and Interferon-gamma (the latter four substances all from Boehringer Mannheim) and a reconstituted homogenate of Sf9 cells expressing Rho baculovirus-derived red blood cell protein 4.1/Pallidin (a kind gift from Dr. Mary Risinger, St. Elizabeth's Medical Center, Boston, MA) were all assessed for nuclease activity by incubation with 1 μ g DNA at 37°C for 4 h and subsequent DNA gel electrophoresis (8). The effect of Ca²⁺, Mg²⁺ and Zn²⁺ ions on nuclease activity was evaluated, and the endo-/exo-nature of the nuclease was determined by the co-incubation of an aliquot of gp120 with the circular plasmid pGEM®-3ZDNA (Promega, Madison, WI, USA).

Cell Culture

Enriched populations of PBMCs were isolated from the blood by density gradient centrifugation. PBMCs were transformed with an anti-CD3 monoclonal antibody (DK-2600; DAKO A/S, Glostrup, Denmark). Where solid-

phase fixation of gp120/anti-gp120 immune complexes was required, goat-anti-rabbit serum (GAR; BIONETICS, Charleston, SC, USA) was used (1). Aliquots of cell suspensions were collected subsequent to various incubation intervals for examination for the presence of apoptotic and necrotic forms and for DNA agarose gel electrophoresis (8).

Detection of Nuclease Activity of Recombinant gp120

Nuclease detection in the gp120-BSA preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to a well-described method (15) with some minor modifications (3,4), and using DNase I (Boehringer Mannheim) with molecular weight of 34 000 Da as a positive control. In addition, because nuclease renaturation of the gp120-BSA preparation (but not the DNase I) proved impossible after SDS-PAGE despite the use of numerous renaturation techniques (3,4), we attempted to detect the nuclease in the gp120 preparation in non-denaturing gels (16–18).

Localization of Nuclease Activity with Respect to gp120 Itself

Immunoprecipitation using Protein-A-Agarose (Oncogene Science, Manhasset, NY, USA) and carried out according to this manufacturer's in-

structions was used to ascertain whether the nuclease activity in the gp120 preparation was functionally distinguishable from the gp120 itself. Fifteen micrograms anti-gp120 antibodies, 4 μ g gp120 and 30 μ L of Protein-A-Agarose were incubated in a microcentrifuge tube at 4°C on a rocking device for 15 h. The immunoprecipitate was collected in a microcentrifuge at 2500 rpm for 15 min at 4°C. The pellet was washed with phosphate-buffered saline (PBS), and then samples of both supernatant and pellet were incubated with 1 μ g DNA for 4 h at 37°C before DNA gel electrophoresis. Aliquots were also submitted to SDS-PAGE electrophoresis, Western blotting, incubation with anti-gp120 antibodies and subsequent detection of gp120 with the peroxidase-4-chloro-naphthol system.

Additional Materials

All other materials and reagents were of analytical grade and were from standard commercial sources.

Figure 2. 72-h supernatants (but not pellets) obtained from cultures of anti-CD3-transformed, HIV- derived, PBMCs cells exposed to crosslinked, solid-phase-fixed gp120 protein (10 μ g/mL) and anti-gp120 (10 μ g/mL) immune complexes, show DNA fragmentation pattern that is the signature of apoptosis. Goat-anti-rabbit serum was used to cross-link anti-gp120 to surface of wells. EP refers to a 15-amino acid peptide representative of the B2 domain of MHC class II (investigation unpublished).

Figure 3. Effect of 1 μ g gp120 suspension on 1 μ g human DNA (lane 1) when co-incubated at 37°C for 4 h and subsequently size-fractionated by DNA agarose gel electrophoresis shows gp120-mediated degradation of the DNA.

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RESULTS

Electrophoretic Assessment of gp120 Nuclease Activity

Resuspended recombinant gp120 (1 μ g) was shown to have nuclease activity since (i) the 72-h supernatants of transformed mononuclear cells derived from HIV- subjects showed the DNA fragmentation pattern that is the signature of apoptosis (Figure 2), and (ii) 1 μ g gp120 suspension incubated with 1 μ g human DNA was shown to degrade the DNA as evidenced by a smear on the electrophoresis gel (Figure 3). The nuclease activity was thermolabile, was Ca^{+2} - and Mg^{+2} -dependent and was inhibited by Zn^{+2} ions (data not shown). Nuclease activity was also endonucleolytic in nature since the gp120 suspension was found to cleave the circular plasmid pGEM-3ZDNA (data not shown). Furthermore, the nuclease activity still exerted detectable effects when used at 0.1 μ g but not at 0.01 μ g.

Electrophoretic Assessment of Nuclease Activity of Recombinantly Produced *E. coli* Proteins

Interferon γ , but not IL-1 α , IL-1 β and IL-6, (all of which are produced recombinantly by *E. coli*), demonstrated nuclease activity (data not shown).

Attempted Detection of Nuclease Activity of gp120 Preparation in SDS-PAGE and in Non-Denaturing Gels

Although numerous attempts at protein renaturation (3,4,13) were made following size-fractionation by SDS-PAGE, we failed to renature the nuclease fraction of the gp120 preparation. However, including DNaseI as a control nuclease that migrated to the correct position (33 kDa) and demonstrated activity after renaturation confirmed that the system was working. Nuclease activity of the gp120 preparation was verified on a non-denaturing gel, but obviously, due to the limitations of this method, the size of the nuclease could not be determined. Data of these experiments were excluded because they were essentially non-helpful.

Immunoprecipitation of gp120 with Subsequent Analysis of Nuclease Activity and the gp120 Content of the Supernatant and Pellet

On immunoprecipitation of gp120 with anti-gp120 antibodies and Protein-A-Agarose, the supernatant, but not the pellet, was found to have the nuclease activity (Figure 4A). Western blot analysis of the pellet demonstrated the presence of gp120, anti-gp120 anti-

bodies and Protein-A-Agarose; whereas, the supernatant lane was empty (Figure 4B).

DISCUSSION

The presence of endonuclease activity in recombinant protein products is clearly of cautionary significance for users investigating putative apoptosis purported to be elicited by such products. In this study, we have demonstrated endonuclease activity in different batch preparations of recombinant gp120 protein, an HIV-1 product incriminated in the pathogenesis of AIDS by virtue of its capacity to prime CD4 T-cells for activation-induced apoptosis (1,5-7,11-13). This process may be aggravated by anti-gp120 antibodies (1) or HIV-1 Tat protein (10,20).

In cultures of cells dying of necrosis, appreciable quantities of DNA are released into the culture medium, where any endonuclease activity is responsible for cleaving the DNA into characteristic 180-200-bp multi-mers, thus simulating death by apoptosis. This appeared to be the case in our study. Similarly, contamination of DNA extracts of normal living cells by trace amounts of culture medium containing endonuclease activity would be expected to have the same effect. Before conclusions can be drawn from the results of culture studies exploring this effect, while not disputing that genuine gp120-induced apoptosis does occur under appropriate circumstances, our findings underline the need for adequate controls in study design to be implemented to exclude the phenomenon we have described.

The identity and molecular weight of the nuclease encountered in our study could not be established, because of its functional sensitivity to the denaturation cycle required for the preparative procedures used. Nevertheless, certain functional characteristics could be inferred indirectly, including its endo-activity, its thermal instability, its Ca^{+2} - and Mg^{+2} -dependence and its Zn^{+2} sensitivity. These characteristics further compound its similarity to the apoptotic endonuclease (2). Immunoprecipitation with Protein-A-Agarose and polyclonal anti-gp120 antibodies of

Figure 4. (A) Immunoprecipitation of gp120 (4 μ g) with anti-gp120 antibodies (15 μ g) and Protein-A-Agarose and subsequent assessment of nuclease activity of the washed pellet and of the supernatant; lane 1: endonuclease buffer plus 1 μ g DNA; lane 2: supernatant plus endonuclease buffer plus 1 μ g DNA; and lane 3: pellet plus endonuclease buffer plus 1 μ g DNA. (B) Western blot analysis of the pellet and the supernatant. Lane 1: anti-gp120 antibodies alone; lane 2: Protein A alone; and lane 3: gp120 protein alone, served as controls for identifying the contents of lanes 4 and 5. Units of protein molecular weight markers are expressed in kDa.

reconstituted gp120 protein, followed by assay of the supernatant and pellet for DNase activity, demonstrated that nuclease activity was confined to the supernatant only. This was consistent with the subsequent Western blot analysis, which showed the gp120 protein to be confined to the pellet and not to present in the supernatant. Thus, the nuclease activity in the gp120 preparation was functionally distinguishable from the gp120 protein itself.

Since Rohrmann's laboratory has reported nuclease activity in wild-type baculovirus-infected cells (14), the presence of similar endonuclease activity in another baculovirus-derived recombinant protein, the red blood cell protein 4.2 (Pallidin) and its absence from most bacterial-derived recombinant protein products studied, suggests that endonuclease contamination of protein products of insect cell expression systems may occur quite frequently. Since the insect cell system is eminently suitable for the production of many comparatively high molecular weight proteins such as cytokines, which may also be undergoing investigation as putative agents responsible for the induction or prevention of apoptosis, this problem could become quite common if not recognized by the investigators.

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