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NMDA and HIV-1 Coat Protein, GP120, Produce Necrotic but Not Apoptotic Cell Death in Human CHP100 Neuroblastoma Cultures via a Mechanism Involving Calpain

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Treatment of neuroblastoma cultures with N-methyl-D-aspartate (NMDA) or human immunodeficiency virus type 1 (HIV-1) coat protein, gp120, induces significant cytotoxic effects which are reduced by leupeptin, E-64, N-Ac-Leu-Leu-norleucinal (ALLnL) as well as by N-Ac-Leu-Leu-normethioninal (ALLnM) and this suggests that activation of the Ca^{2+}-dependent protease, calpain, is involved. The cell death induced by NMDA and gp120 appears to be of the necrotic type; in fact, analysis of DNA fragmentation by flow cytometry or agarose gel electrophoresis failed to demonstrate signs of apoptosis, such as the presence of apoptotic bodies or internucleosomal cleavage. Similar negative results were also obtained by studying the nuclear morphology of the cells with Hoechst 33258 staining. Altogether the data indicate that neuroblastoma cell death induced by NMDA and gp120 is of the necrotic type and this implicates calpain protease.

The human immunodeficiency virus type 1 (HIV-1) coat protein gp120 induces cytotoxic effects in cultured neurones and these seem to be related to its ability to cause an increase in intracellular Ca^{2+} concentration [*see* 1]. Recently, we have reported that selective competitive and non-competitive NMDA receptor antagonists are able to prevent gp120-induced death of human CHP100 neuroblastoma cells in culture; protection has also been afforded by exposing neuroblastoma cultures to gp120 in nominal Ca^{2+}-free medium [2]. These observations support the concept that activation of NMDA subtype of glutamate receptors and the subsequent Ca^{2+} entry through the NMDA receptor-gated cation channel are implicated in the mechanism of cell death caused by the viral protein.

Excessive intracellular Ca^{2+} mediates the ''excitotoxic'' effects induced in neurones by abnormal activation of glutamate receptors [3]. Increase in intracellular levels of this cation activates Ca^{2+}-dependent enzymes, including Ca^{2+}-calmodulin-dependent nitric oxide (NO)-synthase [4] and Ca^{2+}-dependent proteases, such as calpain [5]. Overactivation of these enzymes has been related to excitatory amino acid mediated neuronal damage [6, 7]. Of interest, an excessive production of NO is also involved in the cell death induced by gp120 in rat primary cortical [8] as well as in human CHP100 neuroblastoma [2] cells in culture.

In the present study we investigated the effect of gp120 on the Ca^{2+}-dependent protease, calpain, and its possible involvement in the mechanism of cell death produced by the viral protein in CHP100 neuroblastoma cultures. In addition, since recent *in vitro* [9] and *in vivo* [*see* 10] experimental data demonstrate that excitotoxic neuronal damage produced by abnormal

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stimulation of glutamatergic transmission may also occur through the activation of an apoptotic type of cell death, the possible involvement of apoptosis in the production of NMDA receptor-mediated cell death by gp120 was investigated as well.

MATERIALS AND METHODS

Materials. Recombinant HIV-1 glycoprotein gp120 IIIB (from Baculovirus expression system; >90% pure) was obtained from INTRACEL Corporation (Cambridge, MA, USA). Nicardipine was a gift from Sandoz Prodotti Farmaceutici SpA (Milano, Italy). NMDA was obtained from RBI (Natick, MA, USA); N-Ac-Leu-Leu-norleucinal (ALLnL; calpain inhibitor I), N-Ac-Leu-Leu-normethioninal (ALLnM; calpain inhibitor II) and E-64 were from Calbiochem (San Diego, CA, USA). Leupeptin was obtained from Sigma (St Louis, MO, USA).

Cell cultures and treatments. Human CHP100 neuroblastoma cells were cultured as previously reported [2]. Cells were subcultured from confluent 75 cm^2 flasks and seeded in 35 mm 6-well plates at a density of 75×10^3 cells per well. After culturing for 18-24 hours, the medium was removed and the cultures were washed once with 1 ml ''exposure solution'' (ES) containing (mM): NaCl 120, KCl 5.4, $CaCl_2$ 1.8, Tris HCl 25, glucose 10 (pH 7.4 at room temperature). After 10 min preincubation in the ES at 37°C, NMDA or gp120 were added to the cells for 50 min, then washed off and replaced with normal culture medium for 24 hours prior to evaluate cytotoxicity. For antagonism studies, nicardipine or protease inhibitors were applied to CHP100 cultures 10 min prior to the addition of NMDA or gp120 and they were present during the 50 min exposure time. Each experimental set included determination of cell viability in buffer (control)-, inhibitor-treated cultures and cultures exposed to NMDA and gp120 given either alone or in the presence of the tested drug. Cell viability was assessed by cell exclusion of trypan blue (0.4% w/v) and cell death was reported as the percentage of stained (non viable) vs total cells [2]. The data were expressed as mean±s.e.m. percentage cell death and the resulting means evaluated statistically for differences using the Student's ''t'' test.

Analysis of nuclear morphology by fluorescence microscopy. CHP100 neuroblastoma cells were treated with NMDA (100 μM and 1.0 mM) and gp120 (1.0 and 10 pM) as described above. At the indicated time periods, cell cultures were fixed with acetone:methanol (7:3, v/v) for 5 min, stained with 0.1 μg/ml Hoechst 33258 for 1 hour at 37 °C and observed with a Nikon Diaphot fluorescence microscopy at an excitation wavelength of 365 nm. Apoptotic nuclei stained with Hoechst 33258 have condensed or fragmented chromatin and are highly fluorescent.

DNA gel electrophoresis. Cell pellets (5×10^6 cells) were lysed in 0.5 ml of lysis buffer (5 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% Triton X-100) for 30 min at 4 °C. After centrifugation at 13,000 g for 20 min, DNA from supernatant fractions (low molecular weight DNA) was extracted by the phenol/chloroform method [11], analyzed by electrophoresis in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate/1 mM EDTA) and then visualized by ethidium bromide staining.

Cytofluorimetric analysis. DNA fragmentation was evaluated by flow cytometry on a FACScan flow cytometer (Becton-Dickinson, CA, USA). Cells were fixed in 1 vol of phosphate buffered saline (PBS) plus 1 vol of a methanol:acetone (4:1, v/v) solution at −20 °C and stored at + 4 °C. Cells were incubated with 13 kU/ml ribonuclease A for 15 min at 37°C and then stained with 40 μg/ml propidium iodide at 37°C for 20 min [12]. After staining, cells were excited at 488 nm using a 15 mW Argon laser, and the fluorescence was monitored at 580 nm. Ten thousand events were collected without electronic gating to better show hypodiploic events.

RESULTS

Figure 1 shows that treatment of human CHP100 neuroblastoma cultures with NMDA or gp120 induces significant ($P<0.01$ vs control) cytotoxic effects as evaluated, by trypan blue exclusion assay, 24 hours after a 50 min exposure. The cell death elicited by NMDA (1.0 mM) or gp120 (10 pM) was significantly ($P<0.01$) reduced by the general cysteine protease inhibitor, leupeptin (5.0 μM; n= 5 experiments; Fig. 1A), by E-64 (1.0 μM, n= 5 experiments; Fig. 1B) and by the selective calpain inhibitor, ALLnM (25 μM; n= 7 experiments) (Fig. 1C). Although a slight increase over control cell death was induced by ALLnM given alone (Fig. 1C), at this concentration the protease inhibitor yielded a significant percentage inhibition of NMDA (72.2 ± 10.0%)- and gp120 (54.6 ± 9.8%)-induced cytotoxic effects. A similar (71.1 ± 7.3%; n= 5 experiments) inhibition of NMDA-induced cell death was also afforded by another selective calpain inhibitor, ALLnL (50 μM), though, by comparison, this compound was less effective in reducing gp120-induced cell death (percentage inhibition= 30.6 ± 12.2; n= 5 experiments).

The cell death elicited by NMDA (1.0 mM) and gp120 (10 pM) is sensitive to the blockade

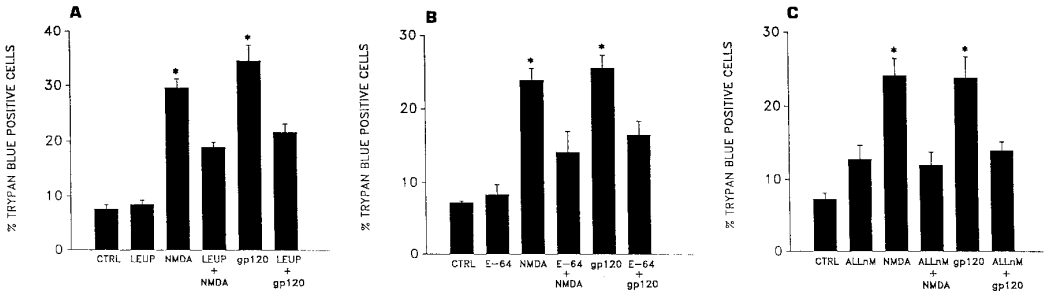

FIG. 1. Exposure of human CHP100 neuroblastoma cultures to NMDA (1.0 mM) and gp120 (10 pM) induces significant cytotoxic effects which are significantly reduced by (A) the general cisteine protease inhibitor, leupeptin (LEUP, 5 μM) which belongs to the peptide aldehyde class of calpain inhibitors, (B) E-64 (1.0 μM), a cysteine protease inhibitor which irreversibly inhibits the Ca^{2+}-dependent protease, calpain, and (C) by the specific calpain inhibitor, N-Ac-Leu-Leu-normethioninal (ALLnM; calpain inhibitor II; 25 μM). The cell death, evaluated as percentage of trypan blue positive cells, was determined 24 hours after 50 min exposure of neuroblastoma cultures to the test compounds. For each protease inhibitor, the data represent the mean ± s.e.m. of 5-7 separate sets of experiments. *Denotes $P<0.01$ vs control (CTRL) cell death and cell death produced in the presence of the protease inhibitor (Student's ''t'' test).

of L-type calcium channels as demonstrated by the complete protection afforded by nicardipine (10 μM), a dihydropyridine Ca^{2+} channel blocker. In these experiments (n= 5), in fact, the percentage cell death values induced by NMDA (25.0±1.9%) and gp120 (23.0±1.6%) were significantly ($P<0.01$) reduced to 7.7±1.2% and 8.1±1.2%, respectively, in the presence of nicardipine, and these were not different ($P>0.05$) from control cell death (6.2±0.9%).

Analysis of nuclear morphology with Hoechst 33258 staining performed at 3, 6, 24 and 48 hours after exposure to NMDA (1.0 mM) and gp120 (10 pM) failed to show the presence of chromatin condensation or fragmentation. As shown in Fig. 2, preservation of a normal nuclear morphology is also apparent in CHP100 cells treated with lower concentrations of NMDA (100 μM) and gp120 (1.0 pM); by contrast, CHP100 cells undergo apoptosis following exposure to high extracellular Ca^{2+} concentrations (10 mM) (Fig. 2, panel F). The data obtained with Hoechst staining are consistent with the findings that analysis of DNA fragmentation by agarose gel electrophoresis (Fig. 3), flow cytometry (Fig. 4) or by TUNEL (deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling; data not shown) failed to demonstrate signs of apoptosis, such as the presence of internucleosomal cleavage or apoptotic bodies, in cells treated with NMDA (100 μM and 1.0 mM) or gp120 (1.0 and 10 pM). In these experiments, exposure of neuroblastoma cells to 10 mM Ca^{2+} was used as a positive control for apoptosis (*see* Figs. 3-4).

DISCUSSION

The results of the present study demonstrate that death of human CHP100 neuroblastoma cells induced by NMDA and gp120 is prevented by four inhibitors of calpain which belong to different pharmacological classes. In fact, the selective calpain inhibitors, ALLnL and ALLnM, as well as the general cysteine protease inhibitor leupeptin, are members of the peptide aldehyde class of calpain inhibitors whereas E-64, which belongs to the epoxysuccinyl peptide class, is able to inhibit irreversibly calpain by forming a sulphide linkage with the active site Cys thiol of the enzyme [*see* 13]. Whilst it has been shown that activation of calpain is implicated in NMDA receptor-mediated neuronal damage both *in vivo* and *in vitro* [6; *see* also refs. in 13], this is the first evidence that activation of this Ca^{2+}-dependent enzyme is involved in the mechanism of cell death produced by gp120. It is conceivable that the mecha-

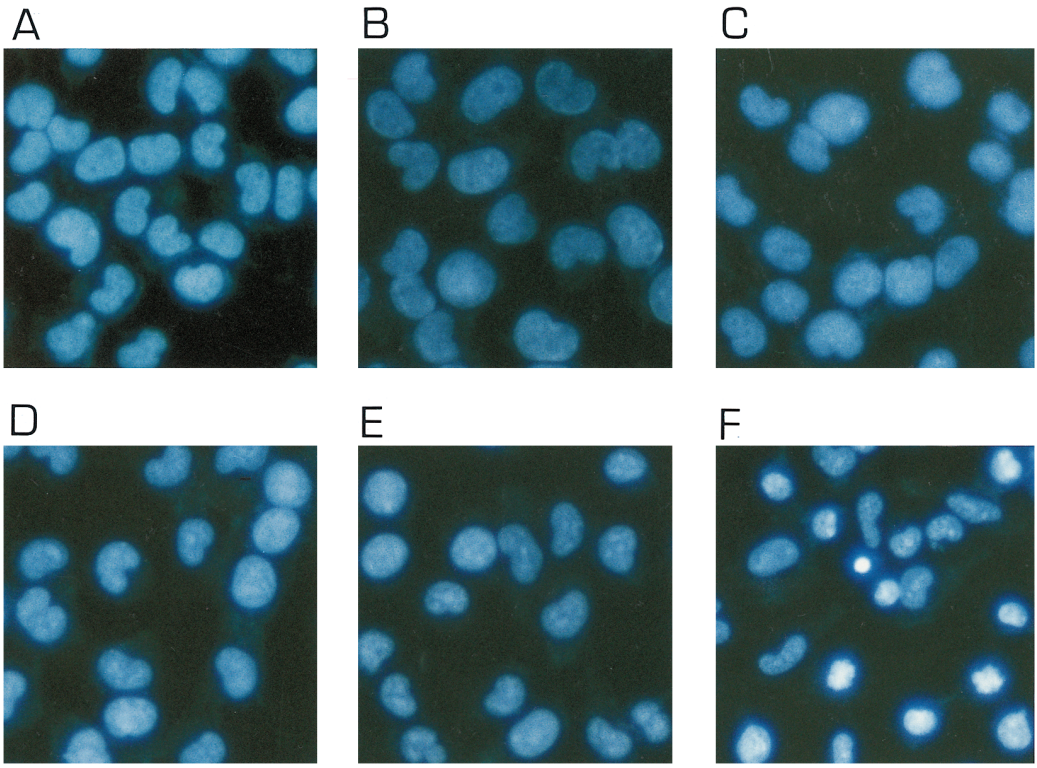

FIG. 2. Morphological analysis of nuclear chromatin in CHP100 neuroblastoma cells stained with the DNA-binding fluorochrome Hoechst 33258 twenty-four hours after exposure to NMDA or gp120. CHP100 cells treated with either NMDA (B, 100 μM; C, 1.0 mM) or gp120 (D, 1.0 pM; E, 10 pM) maintain a normal nuclear morphology; by contrast, in comparison to control cultures (A), cells showing typical condensation of chromatin are evident in cultures exposed to high extracellular Ca^{2+} concentrations (10 mM; F). 20× magnification.

nism through which gp120 activates calpain in CHP100 neuroblastoma cultures involves NMDA receptor-gated Ca^{2+} entry into the cells [2]. In addition, the observation that the dihydropyridine, nicardipine, prevents NMDA- and gp120-induced cell death (present data) indicates that voltage-dependent Ca^{2+} channels may contribute to the raise in intracellular level of this cation and possibly to the activation of calpain.

Evidence exists demonstrating that activation of calpain may be involved in apoptotic cell

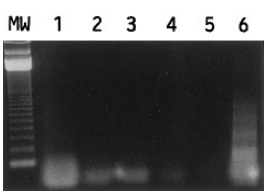

FIG. 3. Agarose gel electrophoresis of low molecular weight DNA isolated from NMDA- and gp120-treated CHP100 neuroblastoma cultures shows that cell death is not associated to DNA fragmentation. Note the occurrence of DNA fragmentation in neuroblastoma cells exposed to high extracellular Ca^{2+} (10 mM) concentrations (lane 6). Lane 1, control (buffer-treated cells); lane 2, 100 μM NMDA; lane 3, 1.0 mM NMDA; lane 4, 1.0 pM gp120; lane 5, 10 pM gp120; M, DNA molecular weight markers. The gel is typical of three individual experiments.

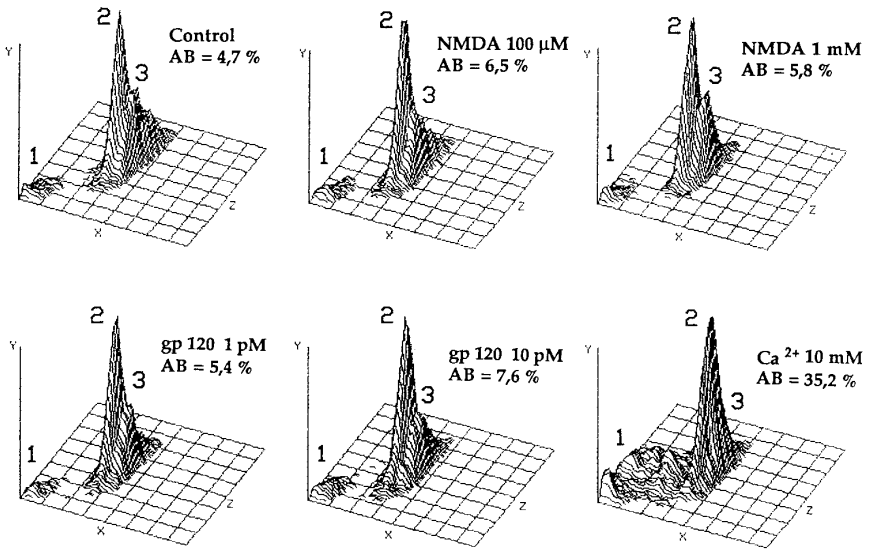

FIG. 4. Three-dimensional evaluation of the cell cycle in CHP100 neuroblastoma cells. In comparison to buffer-exposed cells (Control), a significant percentage of apoptotic bodies (AB) was observed in cells exposed to high extracellular Ca^{2+} (10 mM) but not in NMDA (100 μM and 1.0 mM)- or gp120 (1.0 and 10 pM)-treated cultures. The axes represent 580-nm fluorescent emission by propidium iodide (*x* axis), cell volume (*z* axis) and number of events (*y* axis). The three peaks indicate hypodiploic events (evaluation of apoptotic bodies, AB; peak 1), non proliferating cells (G0; peak 2) and proliferating cells (G2 + M; peak 3). The *x* axis is in a logaritmic scale; for this reason, peaks (2+3) are not very well resolved; this is necessary to better show the peak of interest (1). Each graph represents 10,000 events.

death induced by a variety of agents [*see* 14; but *see* also 15]. However, no biochemical and morphological characteristics of apoptosis were detected in CHP100 cells treated with NMDA (1.0 mM) and gp120 (10 pM); neither evidence for apoptotic cell death was obtained in our present experiments by lowering the concentrations of NMDA or gp120, thus ruling out the possibility of concentration-dependent disclosure of apoptosis [*see* 9]. It has been reported that in rat brain cortical cell cultures gp120 produces DNA laddering, thus favouring an apoptotic type of cell death [16]. In addition, more recent *in vivo* data obtained in our laboratories have demonstrated the occurrence of apoptosis in the brain cortex of rats receiving intracerebroventricular injection of gp120 [17-19]. To reconcile the apparent discrepance between the latter observations and our present data it can be hypothesized that gp120 induced apoptosis via activation of cellular mechanisms which are present in post-mitotic cortical neurones but not in our proliferating tumoral cells. A more attractive hypothesis which could account for our failure to observe apoptosis would be that involving factors originating from cellular elements other than cortical neurones, e.g. microglial cells [*see* 20], obviously not present in neuroblastoma cultures. The observation that CHP100 neuroblastoma cells undergo condensation of nuclear chromatin and DNA fragmentation following exposure to high extracellular Ca^{2+} concentrations (10 mM) does, indeed, confirm the ability of these cells to activate the apoptotic programme of death.

In conclusion, the present data indicate that calpain activation is involved in the necrotic cell death induced by NMDA and gp120 in human neuroblastoma cultures. The mechanism through which calpain leads neuroblastoma cells to death remains to be established. Further experiments are in progress to test whether proteases other than calpain (e.g. ICE) are involved in the cytotoxic effects of NMDA and gp120 and this in relation to a possible role of calpain in the activation of a downstream protease cascade.

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