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Protective Effect of Interleukin-6 against the Death of PC12 Cells Caused by Serum Deprivation or by the Addition of a Calcium Ionophore

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ABSTRACT. Interleukin-6 (IL-6) is known to differentiate the rat pheochromocytoma cell line PC12 to neuron-like cells. We examined the effect of IL-6 on the death of PC12 cells. IL-6 significantly blocked the death of PC12 cells by serum deprivation. The protective effect of IL-6 was increased by preincubation of PC12 with IL-6 for 20 hr before serum deprivation. The inhibition of protein synthesis by cycloheximide had no effect on the protective effect of IL-6 on the serum deprivation-induced cell death. IL-6 also inhibited the death of PC12 cells induced by addition of the calcium ionophore A23187 to the culture medium. Specific *in situ* labeling of DNA cleavage was observed in PC12 cells subjected to both serum deprivation and A23187 for 24 hr. IL-6 inhibited DNA fragmentation in PC12 cells following serum deprivation. These results suggest that the death of PC12 cells induced by serum deprivation or by the addition of calcium ionophore is apoptosis, and that IL-6 blocks apoptosis of PC12 cells. BIOCHEM PHARMACOL 52;6:911–916, 1996.

KEY WORDS. interleukin-6; apoptosis; PC12 cells; serum deprivation; calcium ionophore; cell death

Cell death is a prominent feature of normal neural development. Approximately half of all neurons die during embryonic and immediately postnatal life, and the selection of surviving cells appears to be the result of competition for limited amounts of trophic factors such as NGF‖ [1]. Cell death triggered by withdrawal of trophic support generally appears to follow an apoptotic pathway [2, 3]. Apoptosis is a type of cell death in which morphologic changes include condensation of the chromatin followed by shrinkage of the cell body [4]. Cleavage of cellular DNA occurs through the activation of endogenous endonucleases [3].

In the rat pheochromocytoma cell line PC12, which has been used for studying the mechanism of neuronal cell death, apoptosis can be induced by such conditions as serum deprivation [2, 3, 5], NGF deprivation [6–8], and the addition of a calcium ionophore [9, 10]. These previous studies demonstrated that cell death after NGF withdrawal in neuronally differentiated PC12 cells is retarded by RNA and protein synthesis inhibitors [6–8], while such inhibitors do not prevent the death of undifferentiated native PC12 cells [5]. NGF [2] and FGF [11] prevent the death of PC12 cells caused by serum deprivation. In addition, it has been demonstrated that forskolin and cyclic AMP analogs, as well as IGF-I and IGF-II, rescue PC12 cells from serum-free cell death, and that EGF and chronic depolarization with high K^+ appear to delay rather than prevent cell death [5].

In addition to neurotrophic factors such as NGF, recent evidence suggests that cytokines play an important role in neuronal development, maturation, survival, and regeneration in the nervous system. For instance, IL-1, which is an astroglial growth factor [12], increases neuronal survival in dissociated spinal cord cultures [13]. IL-2 enhances the viability of primary cultured cortical neurons [14]. IL-6 reportedly differentiates PC12 to neuron-like cells [15]. In addition, it has been shown that IL-6 supports the survival of cultured rat CNS cholinergic and catecholaminergic neurons [16, 17], and protects rat hippocampal neurons against glutamate-induced neurotoxicity [18].

In this study, we examined the effects of IL-1, IL-2, and IL-6 on the death of PC12 cells caused by serum deprivation. We show that IL-6 decreased apoptosis of PC12 cells

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‖ *Abbreviations:* NGF, nerve growth factor; IL, interleukin; hIL, human IL; mIL, murine IL; FGF, fibroblast growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; ATA, aurintricarboxylic acid; CYC, cycloheximide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM(+), Dulbecco's modified Eagle's medium with 10% (v/v) fetal bovine serum and 5% (v/v) horse serum; DMEM(−), serum-free DMEM; and LDH, lactate dehydrogenase.

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induced by serum deprivation or treatment with the calcium ionophore A23187.

MATERIALS AND METHODS

Recombinant hIL-6 was a gift from the Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan), recombinant hIL-1α and hIL-1β were gifts from the Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan), and recombinant hIL-2 was donated by the Takeda Chemical Industry (Osaka, Japan). Recombinant mIL-6 and anti-hIL-6-antibody were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). NGF (2.5S, from mucose submaxillary glands), ATA, CYC, and A23187 were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). H-8, KN-62, and H-89 were purchased from the Seikagaku Co. (Tokyo, Japan).

PC12 cells were maintained in DMEM(+). For serum-free experiments, PC12 cells were washed in DMEM(−) by three cycles of centrifugation/resuspension. The washed cells were plated at a density of 1-1.5 × 10^4 cells/cm^2 in a collagen-coated plate. A23187 was dissolved in DMSO diluted with DMEM(+) to achieve a final concentration of 0.1% (v/v).

We examined the effects of various interleukins including hIL-1α, hIL-1β, hIL-2, and hIL-6, on the number of surviving cells after incubation in DMEM(−). Interleukins were added when cells were seeded in DMEM(−). Interleukins were first dissolved in PBS with 0.1% (w/v) bovine serum albumin and then added to the PC12 cell culture. The effects of interleukins were judged according to the number of viable cells as assessed by the MTT assay [19] or by measurement of the release of LDH into the culture medium. In the MTT assay, standard curves representing formazan formation by PC12 cells seeded at various densities were prepared before the assays. LDH was measured with a kit from Boehringer Mannheim Inc. (Mannheim, Germany). For LDH measurement, the culture medium was aspirated, and the cells were treated with 0.1% (v/v) Triton X-100.

The effect of hIL-6 on the survival of PC12 cells was examined when hIL-6 was adsorbed with anti-hIL-6-antibody. Also examined were the influence of duration of incubation with hIL-6 on the protective effect of hIL-6, the effect of CYC, a protein synthesis inhibitor, on the protective effect of hIL-6 against PC12 cell death, and the effect of hIL-6 on the death of PC12 cells induced by the addition of a calcium ionophore, A23187.

We determined whether the cleavage of DNA in PC12 cells occurred under specific conditions by use of an *in situ* apoptosis detection kit, Apoptag, from Oncor Inc. (Gaithersburg, MD, U.S.A.). PC12 cells cultured in DMEM(−) for 24 hr or treated with A23187 (1 μM) for 24 hr in the presence or absence of IL-6 (50 ng/mL) were fixed for 10 min with 10% (v/v) formalin containing PBS. Endogenous peroxidase was inactivated with 20% (v/v) H_2O_2 for 5 min. Residues of digoxigenin-nucleotides were added to DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes the template-independent addition of deoxyribonucleotide triphosphate to the 3′-OH ends of DNA. The incorporated nucleotides form a random heteropolymer of digoxygenin-11-dUTP and -dATP in a ratio that has been optimized for anti-digoxigenin antibody binding. The cells were then incubated with anti-digoxigenin-peroxidase for 30 min. Labeling was revealed with 0.05% (w/v) diaminobenzidine. Lastly, cells were counterstained with methyl green. Apoptotic cells were counted by a computer-assisted image-analysis system (C. Imaging Systems; Compix Inc., Mars, PA, U.S.A.) attached to a light microscope (Olympus BX60-FLB-3; Olympus, Tokyo, Japan).

DNA fragments typical of internucleosomal digestion were detected in the incubating medium as reported previously [9]. Therefore, DNA precipitated from the incubating medium was studied. PC12 cells were incubated in 75 cm^2 dishes that contained 10 mL of medium. Following treatment, the medium from each sample was collected. DNA was precipitated from the medium by a modification of previously established methods [10]. First cellular debris in the medium was removed by centrifugation at 200 *g* for 5 min, and a solution containing 10 mM Tris, 1 mM EDTA, 0.3 M sodium acetate and ethanol (2.5 vol) was added to the supernatant and kept at −20° overnight. The suspension was centrifuged at 10,000 *g* for 30 min, and the pellet was incubated in a solution containing 20 mM EDTA, 10 mM Tris, 0.2% (v/v) Triton X-100 and 200 μg/mL proteinase K for 1 hr at 50°. Samples were sequentially extracted with equal volumes of phenol, phenol/chloroform, and chloroform. The supernatant was again ethanol precipitated and suspended in 10 mM Tris and 1 mM EDTA. The samples were loaded on a 1.2% (w/v) agarose gel containing 0.1 μg/mL ethidium bromide, and subjected to electrophoresis.

Experiments were performed in duplicate and repeated at least three times. The results are expressed as means ± SEM. Statistical significance was determined by one-way ANOVA followed by the two-tailed Bonferroni multiple comparison test, or by Student's *t*-test (two-group comparison). A P value of less than 0.05 was regarded as statistically significant.

RESULTS

When PC12 cells were incubated in DMEM(−) for 48 hr, approximately 70% of them died. hIL-6 significantly blocked the death of PC12 cells induced by serum deprivation, whereas hIL-1α, hIL-1β, and hIL-2 at 25–100 ng/mL had no effect on their survival (Fig. 1). The effect of mIL-6 was almost the same as that of hIL-6 (data not shown). The protective effect of hIL-6 against the death of PC12 cells was negated when hIL-6 was adsorbed with anti-hIL-6-antibody (data not shown).

The addition of hIL-6 to the culture medium 20 hr before serum deprivation and again at the time of serum depriva-

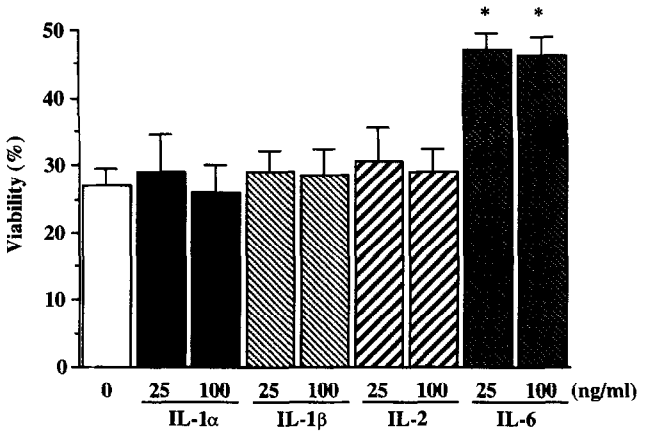

FIG. 1. Effects of interleukins on the death of PC12 cells induced by serum deprivation. PC12 cells were seeded at a density of 0.5 × 10^4 cells/well in a 96-well collagen-coated culture plate. Interleukins were added to DMEM(−) at the time of serum deprivation. Viability of cells was determined by MTT assay 48 hr after serum deprivation. Values are means ± SEM (N = 3–4). Key: (*) $P < 0.01$ vs control.

tion significantly increased its protective effect against the death of PC12 cells. The addition of hIL-6 to the culture medium 20 hr before serum deprivation but not after serum deprivation resulted in only a weak effect on the death of PC12 cells (Fig. 2). Preincubation with hIL-6 for 48 hr yielded no increase over the protective effect achieved by preincubation for 20 hr (data not shown). Figure 3 shows the concentration–response effect of hIL-6 on the death of

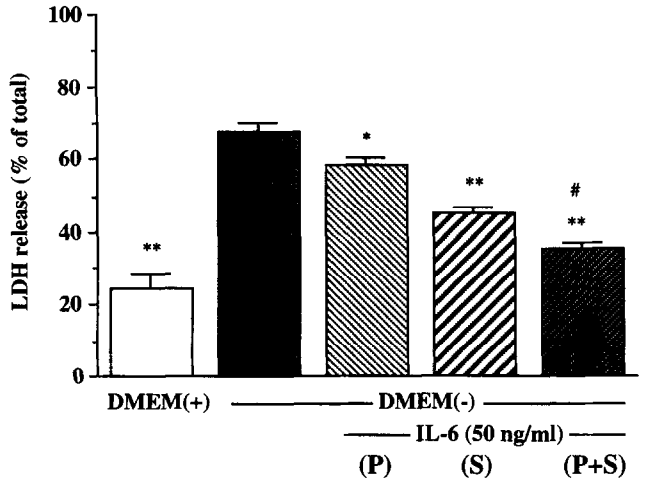

FIG. 2. Influence of duration of incubation with hIL-6 on the protective effect against the death of PC12 cells. PC12 cells were seeded at a density of 3 × 10^4 cells/well in a 24-well collagen-coated culture plate. hIL-6 (50 ng/mL) was added either 20 hr before, but not after, serum deprivation (P), at the time of serum deprivation (S), or 20 hr before serum deprivation and also at the time of serum deprivation (P + S). Viability of cells was determined by LDH assay 48 hr after serum deprivation. Total LDH activity was 0.13 ± 0.02 IU/well. Values are means ± SEM (N = 3–4). Key: (*) $P < 0.05$ and (**) $P < 0.01$ vs serum deprivation alone [DMEM(−)]; and (#) $P < 0.01$ vs treatment S.

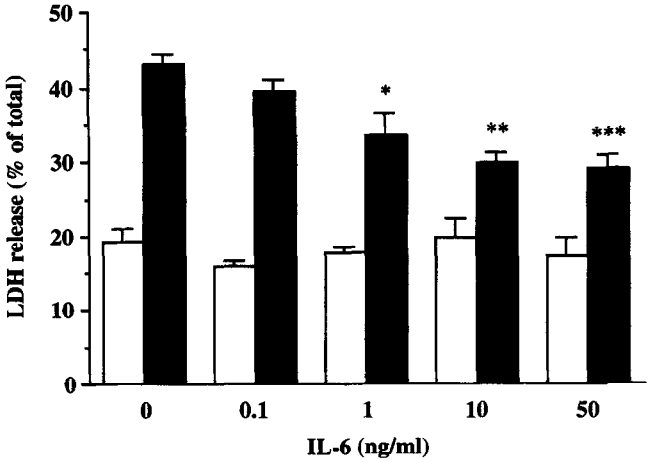

FIG. 3. Concentration–response effect of hIL-6 on the death of PC12 cells caused by serum deprivation. PC12 cells were seeded at a density of 3 × 10^4 cells/well in a 24-well collagen-coated culture plate. hIL-6 was added to the culture medium [DMEM(+)] 20 hr before serum deprivation, and also added to DMEM(−). Viability of cells was determined by LDH assay 48 hr after serum deprivation. Total LDH activity was 0.13 ± 0.02 IU/well. Open column: DMEM(+). Closed column: DMEM(−). Values are means ± SEM (N = 8). LDH release in the presence of any concentration of hIL-6 in DMEM(−) was significantly ($P < 0.01$) higher than that of the corresponding control [DMEM(+)] Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ vs control [DMEM(−)] at 0 ng IL-6/mL.

PC12 cells caused by serum deprivation, under conditions in which hIL-6 was added to the culture medium [DMEM(+)] 20 hr before serum deprivation, and also added to DMEM(−) after serum deprivation. hIL-6 produced a concentration-dependent protection of PC12 cells from cell death caused by serum deprivation; the maximal effect was observed at a concentration of 10 ng/mL. Treatment of PC12 cells with CYC (10 μg/mL), a protein synthesis inhibitor, neither affected the viability of PC12 cells nor prevented the cell death caused by serum deprivation. Moreover, inhibition of protein synthesis did not interfere with the ability of hIL-6 to decrease cell death (Fig. 4).

The addition of NGF (50 ng/mL) or ATA (12.5 μM) to DMEM(−), at the time of serum deprivation, completely blocked the death of PC12 cells induced by serum deprivation. None of the protein kinase inhibitors such as KN-62 (Ca^{2+}/calmodulin-dependent protein kinase II), H-8 (cyclic GMP- and cyclic AMP-dependent protein kinase), and H-89 (cyclic AMP-dependent kinase) at 10 μM blocked the death of PC12 cells induced by serum deprivation (Fig. 5).

The addition of A23187 to the culture medium induced the death of PC12 cells in a concentration-dependent manner. The LC_{50} value of A23187 for PC12 cells was estimated to be approximately 1 μM. When hIL-6 at 50 ng/mL was added to the culture medium 24 hr before the addition of A23187, it significantly decreased the death of PC12 cells induced by A23187 at 1 and 3 μM, but had no effect on the massive cell death caused by 10 μM A23187 (Fig. 6).

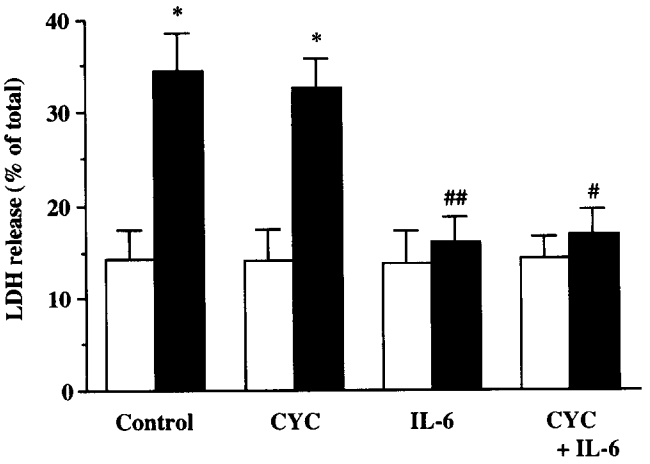

FIG. 4. Effect of CYC on the protective effect of hIL-6 against serum deprivation-induced PC12 cell death. PC12 cells were seeded at a density of 3 × 10^4 cells/well in a 24-well collagen-coated culture plate. PC12 cells were pretreated for 1 hr with CYC (10 μg/mL), and then cultured in the presence of hIL-6 (10 ng/mL) for 20 hr in DMEM(+). Before changing the culture medium to DMEM(−) containing hIL-6 (10 ng/mL), PC12 cells were treated again for 1 hr with CYC (10 μg/mL). Viability of cells was determined by LDH assay 48 hr after serum deprivation. Total LDH activity was 0.13 ± 0.02 IU/well. Open column: DMEM(+). Closed column: DMEM(−). Values are means ± SEM (N = 8). Key: (*) $P < 0.01$ vs corresponding DMEM(+); and (#) $P < 0.05$ and (##) $P < 0.01$ vs control [DMEM(−)].

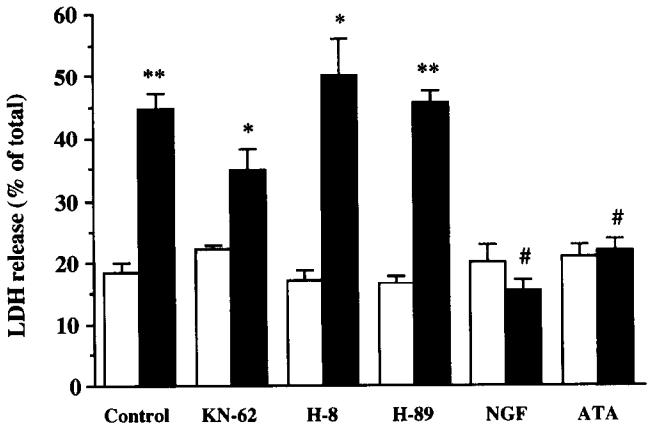

FIG. 5. Effects of various protein kinase inhibitors, NGF, and ATA on the death of PC12 cells induced by serum deprivation. PC12 cells were seeded at a density of 3 × 10^4 cells/well in a 24-well collagen-coated culture plate. KN-62 (10 μM), H-8 (10 μM), H-89 (10 μM), NGF (50 ng/mL), and ATA (12.5 μM) were added to the culture medium at the time of serum deprivation, and viability of cells was determined by LDH assay 48 hr after serum deprivation. Total LDH activity was 0.13 ± 0.02 IU/well. Open column: DMEM(+). Closed column: DMEM(−). Values are means ± SEM (N = 4–8). Key: (*) $P < 0.01$, and (**) $P < 0.01$ vs corresponding DMEM(+); and (#) $P < 0.01$ vs control [DMEM(−)].

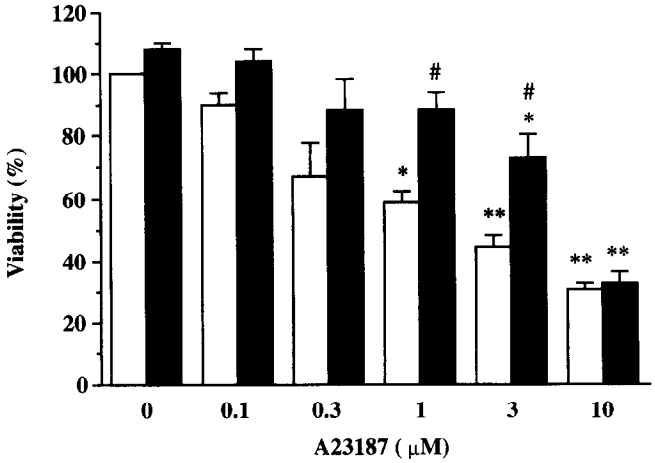

FIG. 6. Effect of hIL-6 against the death of PC12 cells induced by the addition of A23187. PC12 cells were seeded at a density of 0.5 × 10^4 cells/well in a 96-well collagen-coated culture plate. hIL-6 (50 ng/mL) was added to the culture medium [DMEM(+)] 24 hr before the addition of A23187. Viability of cells was determined by MTT assay 24 hr after the addition of A23187. Open column: PC12 cells treated with A23187 alone. Closed column: PC12 cells treated with A23187 and hIL-6. Values are means ± SEM (N = 3–4) Key: (*) $P < 0.05$, and (**) $P < 0.01$ vs corresponding vehicle-treated control; and (#) $P < 0.05$ vs corresponding A23187-treated group.

Apoptotic cells were stained by using the Apoptag kit when PC12 cells were incubated in serum-free medium or with 1 μM A23187 for 24 hr. More than 80% of the cells were stained by serum deprivation and 26% by the treatment with A23187. The number of stained cells decreased significantly when cells were incubated with hIL-6 (50 ng/mL) in serum-free medium (Table 1). We also extracted the DNA of PC12 cells and analyzed it by agarose gel electrophoresis. As shown in Fig. 7, the "ladder-like" pattern of the DNA was evident by serum deprivation (lane 3). hIL-6 inhibited the DNA fragmentation (lane 2) as effectively as NGF did (lane 1).

DISCUSSION

Our present results generally agree with previous findings that the death of PC12 cells following serum deprivation is apoptosis, which is prevented by NGF and ATA, but not by CYC [2, 3, 5]. Furthermore, we showed that various protein kinase inhibitors such as KN-62 (Ca^{2+}/calmodulin-dependent protein kinase II), H-8 (cyclic GMP- and cyclic AMP-dependent protein kinase), and H-89 (cyclic AMP-dependent kinase) had no effect on the serum deprivation-induced death of PC12 cells, suggesting that none of these protein kinases plays an important role.

Among the ILs tested in the present study, we demonstrated that both human and murine IL-6 provided some protection to PC12 cells against apoptotic cell death induced by serum deprivation, as evidenced by the MTT and LDH assays, *in situ* labeling of DNA breaks, and DNA fragmentation in agarose gel electrophoresis. In contrast, other interleukins such as hIL-1α, hIL-1β, and hIL-2 had

TABLE 1. Apoptosis of PC12 cells caused by serum deprivation or by the addition of A23187

Condition	Total number of cells	Apoptotic cells	% of Apoptotic cells
Control			
DMEM(+)	216 ± 13	6 ± 1	3.0 ± 0.5
+A23187 (1 μM)	181 ± 31	47 ± 6*	26.0 ± 1.6*
Serum deprivation			
DMEM(−)	163 ± 19	135 ± 18*	82.7 ± 3.9*
+ IL-6 (50 ng/mL)	197 ± 27	54 ± 10†	27.4 ± 1.6†

In situ labeling of DNA breaks in PC12 cells, caused by the addition of A23187 or by serum deprivation, was performed, and the apoptotic cells were counted as described in Materials and Methods. PC12 cells were stained 24 hr after the addition of A23187 or serum deprivation. IL-6 was added 24 hr before serum deprivation. Values are means ± SEM (N = 4).

* $P < 0.01$ vs DMEM(+).

† $P < 0.01$ vs DMEM(−).

no effect. IL-6 inhibited the appearance of DNA "ladder" as effectively as NGF did. Since the protective effect of hIL-6 on the survival of PC12 cells was negated when hIL-6 was adsorbed with anti-hIL-6-antibody, the protective effect against the death of PC12 cells appears to be specific to IL-6. The protective effect of IL-6 on the apoptotic death of PC12 cells caused by serum deprivation was concentration related, but relatively weak, when compared with the effects of NGF, FGF, IGF-I, and IGF-II. It is feasible that IL-6 may delay rather than prevent the death of PC12 cells caused by serum deprivation, as do both EGF and a high concentration of K^+ [5].

When hIL-6 was added to the culture medium before serum deprivation, its protective effect was increased significantly. This seems important to an understanding of the mechanism of the protective effect. It is unlikely that IL-6 may produce induction of protein synthesis that blocks apoptosis in PC12 cells since the protective effect of IL-6 was not affected by the blockade of protein synthesis by CYC. Therefore, it is suggested that IL-6 decreases apoptosis of PC12 cells caused by serum deprivation by a mechanism that is independent of protein synthesis, as NGF does [5].

We also found that IL-6 inhibits PC12 cell death caused by treatment with the calcium ionophore A23187. A23187 reportedly causes a concentration-dependent increase in cytoplasmic calcium and internucleosomal digestion of DNA in PC12 cells [9]. In the present study, A23187 induced the death of PC12 cells in a concentration-dependent manner, and the occurrence of DNA cleavage was ascertained with the Apoptag kit. It follows, then, that the death of PC12 cells induced by addition of a calcium ionophore may also be apoptosis. Thus, our study suggests that IL-6 inhibits apoptosis induced by either serum deprivation or a calcium ionophore. In some types of apoptosis, the intracellular concentration of calcium changes [20]. A calcium ionophore raises the cytoplasmic concentration of calcium, which is thought to be a trigger of the signal transduction of apoptosis. IL-6 inhibited the death of PC12 cells induced by addition of a calcium ionophore to the culture medium, suggesting that the effect of IL-6 occurs at a step subsequent to an increase of intracellular calcium in

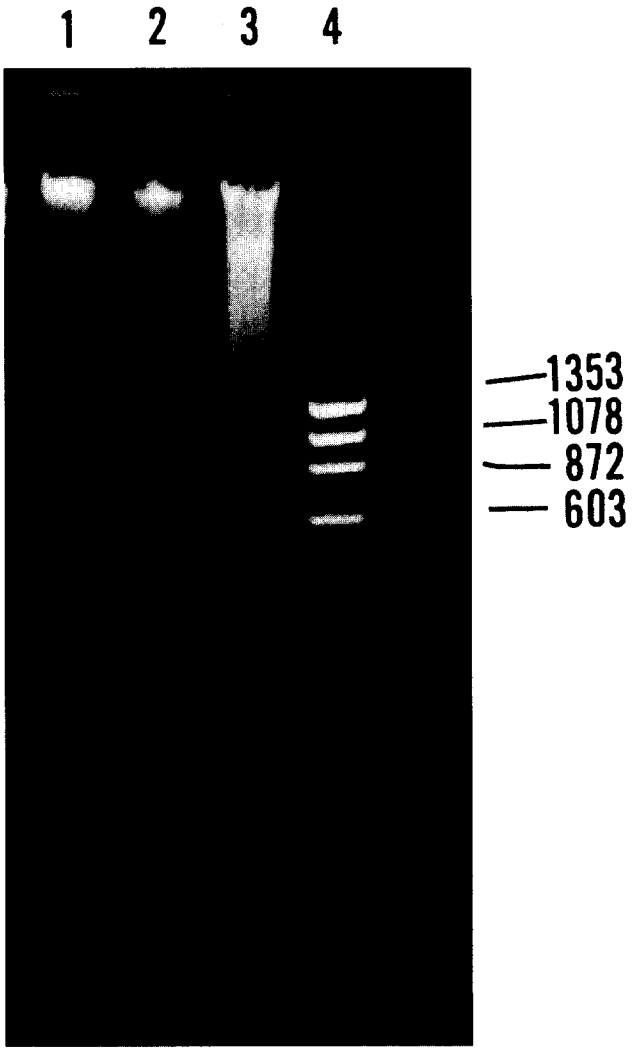

FIG. 7. Agarose gel electrophoresis of DNA. All samples were extracted from the incubating medium. PC12 cells were incubated in DMEM(−) for 14 hr. The numbers on the side of the gel indicate base pairs. Lane 1: added NGF (50 ng/mL); lane 2: added hIL-6 (50 ng/mL); lane 3: no additives; and lane 4: DNA standard. The typical DNA ladder pattern is seen only in lane 3.

the signal transduction of apoptosis. In the case of the death of PC12 cells induced by serum deprivation, however, cytoplasmic calcium may not be involved, since it has been reported that the intracellular concentration of calcium does not change [21], and that extracellular calcium is not required [22], when PC12 cells die by serum deprivation. Taken together, IL-6 appears to inhibit apoptosis, whether or not initiated by an increase of calcium. Further studies should be carried out to clarify the mechanism by which IL-6 blocks the signal transduction of apoptosis. In conclusion, IL-6 partially blocks apoptosis of PC12 cells induced by serum deprivation or by the calcium ionophore A23187.

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