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Glutamate Triggers Internucleosomal DNA Cleavage in Neuronal Cells

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SUMMARY: Glutamate neurotoxicity is responsible for neuronal loss associated with numerous obstinate disorders. In this report, the mechanism of glutamate neurotoxicity was investigated on a viewpoint of DNA degradation. We found that chromosomal DNA of cultured neurons was degraded into nucleosomal-sized DNA fragments by the addition of glutamate, prior to the glutamate-induced neuronal death. Both the neuronal death and DNA fragmentation were prevented by the inhibitors of endonucleases and mRNA synthesis. Furthermore, an injection of glutamate into the rat hippocampi resulted in DNA fragmentation with the similar time course observed in neuronal death *in vitro*. These results suggest that the glutamate neurotoxicity involves an active suicide process which leads to neuronal death through internucleosomal DNA cleavage.

Glutamate is a major excitatory neurotransmitter in mammalian central nervous system (1,2,3,4) and, under pathologic conditions, works as a neurotoxin (5). Considerable attention has been focused on the mechanisms of the glutamate neurotoxicity because it has been claimed to be involved in neuronal loss associated with numerous obstinate disorders - ischemia (6,7), epilepsy (8), Parkinson's disease (9), Huntington's disease (10), and Alzheimer's disease (11). Despite the clinical and biochemical importance of the glutamate neurotoxicity, the understanding of subcellular mechanism of glutamate neurotoxicity is still limited.

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Abbreviation: AT, Aurintricarboxylic acid.

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The purpose of this paper is to investigate the mechanism on the viewpoint of DNA degradation. The dissociated cortical neuron cultures of rat, which were cultured in the chemically defined medium without glutamate, were prepared to examine the effect of glutamate addition. We found that glutamate induces internucleosomal DNA cleavage which ultimately results in the neuronal death. This type of DNA degradation is originally found in programmed cell death of thymocytes induced by glucocorticoids (12,13). Our observations imply that an active suicide mechanism operates in the glutamate-induced death in neuronal cells.

Materials and Methods

Neuronal culture and determination of viability. The cerebral neocortices were removed from fetal Wistar rats (15-16 day gestation) and subjected to the dissociated neuron cultures by the established technique (14). The dissociated cells were plated on poly-L-lysine (Sigma) coated 35 mm dishes (1 x 10^6 cells per dish) in Dulbecco's modified Eagle medium (Gibco) supplemented with 10 μg/ml insulin (Sigma), 10 μg/ml transferrin (Sigma), 10 ng/ml sodium selenite (Sigma), 10 nM progesterone (Sigma), 1 nM triiodothyronine (Sigma), 1 mM sodium pyruvate (Gibco), and 24 mM glucose. Neuronal cultures were maintained for 3 days at 37°C in a humidified 5% CO_2 atmosphere. Thereafter, glutamate was added to medium at final concentration of 1 mM. Viable cortical neurons were identified under a phase-constant microscope by exclusion test of trypan blue.

DNA Fragmentation *in vitro*. High molecular weight DNA was prepared from two dishes of the cultured neurons by the following method. The cultured cells were digested in 1 ml of 10 mM tris-HCl (pH 7.5) containing 30 mM EDTA, 100 mM NaCl, 0.1% SDS, 200 μg ml^{-1} proteinase K (BRL) at 55°C overnight followed by treatment with 0.5 mg ml^{-1} RNase A (Sigma) for 1 h. The digested samples were extracted once with an equal volume of phenol saturated with 10 mM tris-HCl (pH 8.0) buffer containing 1 mM EDTA. The DNA was mixed with 1 ml of isopropanol and 0.1 ml of 3 M sodium acetate (pH 5.2) and subsequently precipitated by centrifugation. Ten micrograms of DNA sample were subjected to electrophoresis on 1.5% agarose gel containing 0.5 mg ml^{-1} ethidium bromide.

DNA fragmentation *in vivo*. The glutamate was topically injected into the hippocampal CA1 subfield of adult Wistar rat. In brief, 2 μl of 20 mM of glutamate (pH 7.5) was administered over a period of 5 min at two points in each side of the hippocampus. Stereotaxical coordination was according to the atlas of Paxnos and Watson (1986). At various time points after the injection, bilateral hippocampi were dissected for the DNA analysis. The hippocampal tissues were homogenized in 2.5 ml of 10 mM tris-

HCl (pH 7.5) containing 30 mM EDTA and 100 mM NaCl with a Dounce-type homogenizer. After the addition of 25 µl of 10% SDS and 50 µl of 10 mg ml^{-1} proteinase K (BRL), the homogenates were incubated at 55°C overnight. Further purification and electrophoresis of DNA were performed as described above.

Other materials. Aurintricarboxylic acid and actinomycin D were purchased from Sigma. Other chemical compounds were regent grade or better.

Results and Discussion

To determine the time course of the toxic action of glutamate in dissociated culture of rat cortices, we examined the viability of the neurons by a trypan blue exclusion test at various times after the addition of 1 mM glutamate. The neuronal death increased exponentially and 46% of the neurons were dead 24 hours after the addition of glutamate (Fig. 1A). In this course of the neuronal death, we found that chromosomal DNA was degraded into integral multiples of nucleosomal-sized fragments (190 base pairs). The pattern of DNA degradation indicates that the chromosomal DNA is digested at internucleosomal-linker regions by activated endogenous endonucleases (15). No DNA fragmentation was observed when neurons were killed by azide or heating (data not shown). Thus, DNA fragmentation seemed to be selective to glutamate-induced neuronal

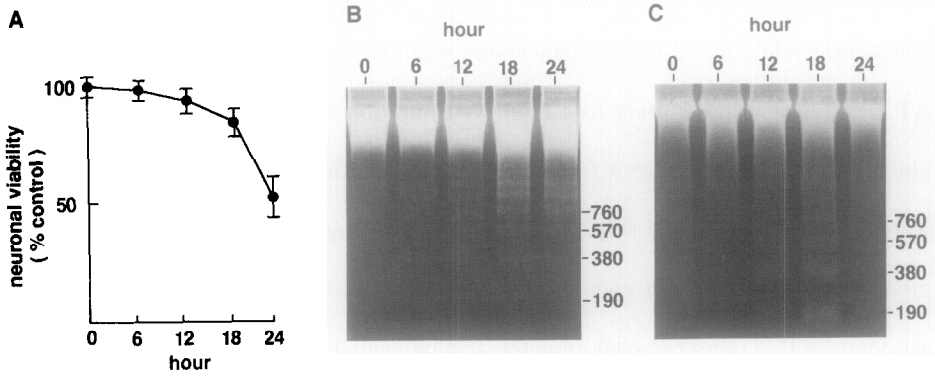

Figure 1. (**A**) Time course of glutamate-induced neuronal death in rat cortical cultured neurons. Neuronal viability was presented as percent (mean ± S.D., n = 4) of that of the sister cultures without glutamate treatment. (**B**) Time course of glutamate-induced DNA fragmentation in cortical cultured neurons. (**C**) Time course of glutamate-induced DNA fragmentation in rat hippocampus. No DNA fragmentation occurred when equal volume of saline was injected instead of glutamate (data not shown).

death. The amount of the fragmented DNA increased gradually, reached a maximum at 12-18 hours after the addition of glutamate, and then declined (Fig. 1B). The neuronal DNA fragmentation required a longer maturation period than that of thymocytes induced by glucocorticoids; DNA fragmentation in thymocyte is evident within 6 hours following the glucocorticoid treatment (13). The mechanism of endonuclease activation in neuron may differ from that in thymocyte.

Aside from glutamate-induced DNA fragmentation, it should be noted that a certain amount of neuronal DNA fragmentation constantly occurred during the culture without any treatment. This "background" fragmentation increased with time and paralleled spontaneous neuronal loss in the culture. Since spontaneous DNA fragmentation made it difficult to estimate the effect of glutamate, particularly in the older cultured neuron, the neuronal culture at day 3 was used throughout this study, despite the previous observation that the older neurons become more sensitive to glutamate (16).

DNA fragmentation preceded to the neuronal death, when compared with the neuronal survival rate (Fig. 1A and 1B). This observation suggested that DNA cleavage may not be a resultant but a causal event of the neuronal death. To clarify the causal relationship between DNA fragmentation and neuronal death, the effect of aurintricarboxylic acid (AT), a general inhibitor of endonucleases (17,18), was examined whether the inhibition of DNA fragmentation resulted in neuronal survival or not. When AT was added to the culture to a final concentration of 100 μM at 2 hours after the glutamate addition, both DNA fragmentation and neuronal death induced by glutamate were remarkably inhibited (Fig. 2A and 2B), which suggests that DNA fragmentation produced by the activated endonucleases leads to the neuronal death. Furthermore, actinomycin D, a transcriptional inhibitor, also prevented the DNA fragmentation and the neuronal death, when it was added to the culture 12 hours prior to the addition of glutamate (Fig. 2C and 2D). Post-treatment with actinomycin D was, however, less effective in limiting glutamate neurotoxicity. The results suggest that the activation process of endonuclease requires gene expression.

To determine the effect of glutamate on the neuronal DNA fragmentation *in vivo*, 160 nmol of glutamate was injected into the bilateral CA1 subfields of rat dorsal hippocampus. DNA extracted from those regions was analyzed at various times after the injection. DNA fragmentation also took place *in vivo* with the similar time course observed in the neuronal death *in vitro* (Fig. 1C). Therefore, DNA fragmentation may be responsible for neuronal loss associated with the glutamate neurotoxicity *in vivo*.

Little is known about the activation mechanism of the endogenous endonucleases. However, increased intracellular Ca^{2+} concentration seems to be important, as evidenced by the fact that the Ca^{2+} ionophore A23187, instead of glutamate, could induce DNA fragmentation (unpublished observation). This view is further supported by another observation that glutamate subtype agonist N-methyl-D-aspartate, whose receptor is closely associated with a cationic channel (19), caused larger magnitude of DNA fragmentation than glutamate (unpublished observation). The behavior of protein kinase C in response to increased Ca^{2+} concentration plays a pivotal role in the operation of an active suicide program in thymocytes (20). Further study is required to clarify the roles of Ca^{2+} influx and protein kinase C in the activation process of neuronal endonucleases.

It is well established that during normal development of the nervous system, large numbers of neurons degenerate and die through a process known as naturally occurring neuronal death (21). Since the naturally occurring neuronal death is prevented by the inhibitors of protein and mRNA synthesis, an endogenous genetic program for suicide is proposed to participate in this type of the neuronal death (22,23). In those reports, they supposed the existence of "killer proteins" which were newly synthesized during the suicide process. The results presented here imply that the suicide program also operates in glutamate-induced neuronal death. It is, therefore, conceivable that programmed cell death is a common mechanism responsible for cell deletion in nervous system. The endonuclease may be a candidate for the "killer proteins" which works in this mechanism. Finally, glutamate can switch neuronal suicide program "on", which has been in sleep from developmental period. The scheme illustrated here may provide a new insight into

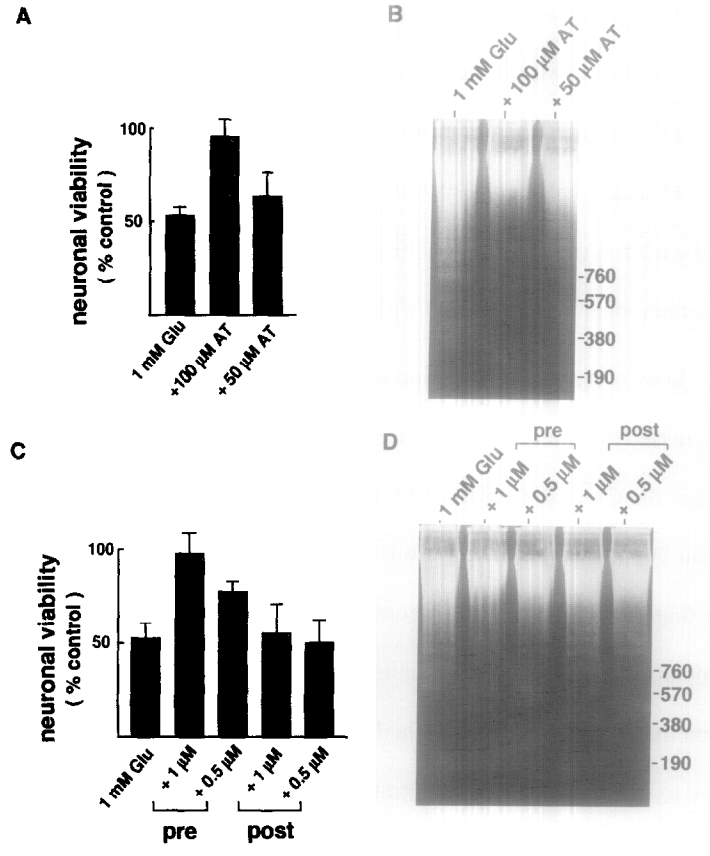

Figure 2. (**A**) Effect of aurintricarboxylic acid (AT) on glutamate-induced neuronal death. AT was added to the culture medium at final concentration of 0.1 or 0.05 mM 2 hours after the glutamate addition. (**B**) Effect of AT on neuronal DNA fragmentation. (**C**) Effect of actinomycin D on glutamate-induced neuronal death. Actinomycin D was added to the culture at indicated concentrations 12 hours before or 2 hours after the addition of glutamate. (**D**) Effect of Actinomycin D on neuronal DNA fragmentation. The neuronal viability (**A** and **C**) and the DNA fragmentations (**B** and **D**) were examined at 24 hours and 18 hours after the addition of glutamate, respectively.

the mechanism underlying the human neurological disorders related with the glutamate neurotoxicity.

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