Mechanisms underlying neuronal death induced by chromogranin A-activated microglia

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Running Title: neuronal apoptosis induced by CGA-activated microglia

Key Words: neuronal apoptosis, chromogranin A, microglia, MAP kinase, caspases, FasL/Fas, cytochrome c
ABSTRACT

The neurotoxic effects of activated microglia in neurodegenerative diseases are well established. We recently provided evidence that chromogranin A (CGA), a multifunctional protein localized in dystrophic neurites and in senile plaques, induces an activated phenotype and secretion of neurotoxins by rat microglia in culture. In the present study, we focused on the mechanisms underlying neuronal degeneration triggered by CGA-activated microglia. We found that neuronal death exhibits apoptotic features, characterized by the externalization of phosphatidylserine and the fragmentation of DNA. Microglial neurotoxins markedly stimulate the phosphorylation and activity of neuronal p38 mitogen-activated protein kinase (MAP kinase), and provoke the release of mitochondrial cytochrome c which precedes apoptosis. Inhibition of p38 kinase with SB 203580 partially protects neurons from death induced by CGA-activated microglia. Furthermore, neurons are also protected by Fas-Fc, which antagonizes the interactions between the death receptor Fas and its ligand FasL and by cell permeable peptides that inhibit caspases-8 and -3. Thus, CGA triggers the release of microglial neurotoxins that mobilize several death-signalling pathways in neurons. Our results further support the idea that CGA, which is up-regulated in many neuropathologies, represents a potent endogeneous inflammatory factor possibly responsible for neuronal degeneration.
INTRODUCTION

Destruction of neurons by apoptosis and necrosis is the underlying mechanism in a variety of neurodegenerative diseases, and thus represents an area of intense interest (1, 2). Activated microglial cells are capable of releasing cytotoxic agents, including cytokines, complement proteins, proteolytic enzymes, nitric oxide and reactive oxygen intermediates, and NMDA-like toxins (3-7). Therefore, activation of microglia is considered to contribute to neuronal injury and has important pathogenic implications in neurodegenerative diseases such as Alzheimer's disease, Parkinson disease, or immunodeficiency virus-associated dementia (4, 6, 8, 11). Despite their clinical importance, the brain-derived factors which control microglial activation and the precise intracellular mechanisms involved in microglial cytotoxicity remain elusive. The main constituent of amyloid plaques, β-amyloid protein, is believed to play a causal role in the pathogenesis of Alzheimer's disease and other neuropathological disorders (9-11). Studies with synthetic peptides have shown that β-amyloid protein is directly toxic to cultured neurons (12, 13), and also induces the production of proinflammatory cytokines in microglia (8-11). However, the initial events that precede the deposit of the neurotoxic form of β-amyloid protein, as well as the direct contribution of activated microglia to the execution of neuronal apoptosis are not clear. Understanding these steps may therefore be relevent for the identification of novel therapeutic strategies in treatment of neurodegenerative disorders.

Chromogranin A (CGA) is a polypeptide chain of 431-445 amino acids corresponding to a 48-52 kDa glycoprotein (14), which is widely distributed in endocrine and nervous tissue. In all
tissues examined so far, CGA is proteolytically processed into peptides, of which some have defined biological activity (14, 20, 21). For instance, CGA-derived peptides have been shown to regulate secretion in various endocrine cell types (22-24), and to modulate adhesion and spreading of fibroblasts (25). CGA and its proteolytic fragments have been found, together with β-amyloid protein, in senile and pre-amyloid plaques (11, 15-17). Moreover, CGA has been detected in large dystrophic neurites containing the amyloid precursor protein (15, 18, 19), suggesting that it may be one of the endogeneous factors released from damaged neurons into brain deposits. We have previously described that CGA is able to induce an activated phenotype in cultured microglia, characterized by changes in morphology and actin organization, generation of nitric oxide, secretion of TNF-α and production of heat-stable diffusible factors that provoke neuronal degeneration (26, 27). A recombinant N-terminal fragment of human CGA, vasostatin I corresponding to residues 1-78, stimulates secretion of microglial neurotoxins to a similar extent, suggesting that the active domain is present in the N-terminal region of the CGA molecule (27).

In the present study, we focused on the mechanisms underlying neuronal death induced by CGA-activated microglia. We found that the death-domain containing receptors, Fas/Apo1/CD95, that belong to tumor necrosis factor receptor family (29) and the mitogen-activated protein kinase p38 are possible transductors of the neuronal response to microglial neurotoxins. The pathways mobilized in neurons also include release of mitochondrial cytochrome c (28) and activation of caspases-8 and -3. These results further supports the idea that CGA represents a potent brain endogenous inflammatory factor, capable of inducing the production of microglial neurotoxins responsible for the mobilization of several apoptotic cascades within neurons.
MATERIALS AND METHODS

Purification of bovine chromogranin A (CGA)

CGA was purified from bovine adrenal medullary chromaffin granules as described by Simon et al. (30) with an additional purification step on a column of Ultraspherogel Sec 2000 (7.5 x 30 mm, Beckman). Stock solutions usually containing 50 µM CGA were aliquoted and stored at -20°C. The final CGA preparation was composed of the native 70 kDa CGA (60 % of the total proteins) and smaller processed components (60 to 43 kDa) together representing 40 % of the total protein as estimated from scanned monodimensional electrophoretic profiles. Immunoblotting indicated that all protein bands were immunoreactive with specific anti-native CGA antibodies (31) and anti-CGA[124-143] antibodies (30, 31). Sequence analysis (by automatic Edman degradation on an Applied Biosystems 473A microsequencer) demonstrated that the final CGA preparation contained only CGA-derived sequences with 80 % of the sequenced material containing the N-terminal sequence of CGA (L-P-V-N-S-). The activity of the CGA preparations was systematically controlled by measuring the production of NO in microglial cell cultures as described previously (26, 27).

The content of endotoxin in the CGA preparations was determined at the Institut d'Hygiène et de Médecine Préventive (Strasbourg, France) with the chromogenic Limulus amoebocyte lysate test (Coamatic Endotoxin Chromogenix, Biogenic, Maurin, France). At 10 nM, the CGA preparations contained less endotoxin than the culture medium alone, which itself is unable to trigger the NO production in microglial cells. CGA was usually dissolved in serum-free defined medium and applied to cultures by changing the medium at the indicated concentrations.
Neuronal cultures

Neuronal cell cultures were established after mechanical dispersion of cerebral cortices from Wistar foetal (day 14 of gestation) rat brain as previously described (27). Briefly, cerebral cortices cleaned from meninges were forced through a nylon sieve (pore size 48 µm) in nutrient medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum. The cell suspension was centrifuged and cell viability determined with trypan blue. Viable cells were plated at a density of 2.5 x 10^5 cells per well in 24 multiwell Falcon plates coated with poly-L-ornithine in DMEM supplemented with 10% foetal calf serum. For immunocytochemistry, each well contained a 12 mm glass coverslip. Cells were subsequently incubated for 1 hour at 37°C in a 5% CO₂ humidified atmosphere. The serum-containing medium was then replaced by serum-free defined medium consisting of DMEM supplemented with transferrin (100 µg/ml), insulin (5 µg/ml), albumin (100 µg/ml), progesterone (6 ng/ml) and sodium selenite (5.2 ng/ml).

Microglial cultures

Microglial cells were isolated from high-density glial cell cultures as previously described (26). Briefly, cells dissociated from cerebral hemispheres of neonatal rat brain (Wistar strain) were plated at a density of 5 x 10^4 cells/cm² in culture medium consisting of DMEM supplemented with 10% foetal calf serum. Culture medium was changed after 5 days and then twice a week. After 2 weeks, cultures contained glial cells including amoeboid microglia mostly localized on the top of the cellular layer. The loosely adherent microglial cells were recovered by shaking. After centrifugation (100 g/5 min), cell viability was determined by trypan blue.
exclusion, and viable cells were then plated at a final density of $2.5 \times 10^5$ cells per well on 24 multiwell Falcon plates in DMEM supplemented with 10% foetal calf serum. Non-adherent cells were removed 30 min after plating by changing the medium for serum-free defined medium. Amoeboid microglia isolated from neonatal rat brain cultures consist of flat, round or spindle-shaped, highly adherent cells. They respond to bacterial endotoxin (32) and express Fc (33) and CR3 complement receptors (26).

Conditioned medium was prepared by incubating microglial cell cultures for 24 hours in serum-free defined medium with or without 10 nM CGA. Medium was subsequently collected and cleared by centrifugation (1000 g/20 min). When necessary, the medium was stored at -20°C and used either non-diluted or diluted to 80% with serum-free defined medium. In all experiments, conditioned medium from CGA-untreated and CGA-treated microglia were collected simultaneously and used in parallel.

**Neuron/Microglial co-cultures**

Co-cultures of neurons and microglial cells were prepared by plating a suspension of isolated microglia ($2.5 \times 10^5$ cells) on neurons maintained 4 days in culture. Cells were incubated for 30 min in DMEM containing 10% foetal calf serum. Serum-free defined medium with or without additives was then added and co-cultures were grown for at least 6 days.

**Treatment of cultures**

Neurons were grown for 4 days before being incubated with conditioned media from CGA-untreated or CGA-treated microglia for the indicated period of time. Recombinant human APO-1/Fas-Fc IgG (Fas-Fc), soluble Fas ligand (sFasL) with enhancer antibodies (Alexis Corp.,
Coger, Paris, France) were diluted in the indicated culture medium. Inhibitors of caspases, Z-DEVD-FMK (Z-Asp-Glu-Val-Asp-fluoro methyl ketone) and Z-IETD-FMK (Z-Ile-Glu-Thr-Asp-fluoro methyl ketone), (Calbiochem, Meudon, France) diluted in DMSO were used at the indicated concentrations. Controls were performed in the presence of the same concentrations of DMSO.

**Assay of viability and apoptosis**

Neuronal survival in living cultures was estimated by incubating cells at 37°C for 10 min with 2.5 µg/ml nuclear fluorochrome propidium iodide (PI, Sigma, St Quentin, Fallavier, France). Alternatively, cultures were incubated for 5 min with 0.1% trypan blue in PBS and stained cells were immediately counted in three randomly chosen fields.

Nuclei in paraformaldehyde fixed cells were visualized by staining with Hoechst 33342 (Sigma, St Quentin, Fallavier, France) for 10 min at a final concentration of 2.5 µg/ml. Cultures were examined with a microscope equipped with an epifluorescence system and appropriate filters (Zeiss Axioscope, Carl Zeiss, Oberkochen, Germany) and those neurons with clearly condensed and segmented chromatin were counted as apoptotic.

Binding of annexin V was performed by incubating living cells (30 min, room temperature) with fluorescein-conjugated annexin V (Bioproducts, France) diluted 1/30 in serum-containing culture medium in the presence of 0.01% sodium azide. After washing and fixation, cultures were further processed for immunocytochemistry.

DNA fragmentation was detected in fixed cells using the “Apoptosis Detection System, Fluorescein” kit (Promega, France) by incorporating fluorescein-12-dUTP at the 3’-OH ends of the DNA using terminal deoxynucleotidyl transferase, according to the manufacturers
instructions.

**Immunocytochemical staining and confocal laser scanning microscopy**

Cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min, followed by a 5 min permeabilization with 0.02% Triton X-100 in 4% paraformaldehyde. Cultures were then incubated at room temperature for 30 min in phosphate-buffered saline (PBS) containing 10% bovine serum albumin to inhibit nonspecific binding before a 1 hour incubation with primary antibodies. Monoclonal antibodies to microtubule-associated protein 2 (MAP2, SMI 52, Sternberger Monoclonals Incorporated) were used at 1/500 dilution. Monoclonal anti-cytochrome c antibodies (PharMingen Europe, clone 6H2.B4) were used at 1/500 dilution. For detection of FasL with rabbit polyclonal antibodies (N-20 or C-178, Santa Cruz, CA), living cells were incubated with 0.1-0.2 µg/ml of IgG in serum-containing culture medium in the presence of 0.01% sodium azide at room temperature for 30 min and then fixed. After washing with PBS (5 changes, 30 min), the coverslips were incubated for 1 hour with either fluorescein (FITC)- or rhodamine (TRITC)-conjugated affinity purified F(ab')2 fragment of goat anti-mouse class G immunoglobulins used at 1/300 dilution (Fc fragment specific, Jackson Immunoresearch Laboratories, West Grove, PA/USA). Coverslips were subsequently washed with PBS (5 changes, 30 min) and mounted in Mowiol (Calbiochem, Meudon, France).

Mounted coverslips were examined with a Zeiss Axioscop microscope equipped with an epifluorescence system and appropriate filters. Sequential through-focus images of labelled cells were obtained using a Zeiss laser scanning confocal microscope (LSM 410 invert) equipped with a planapo oil (63x) immersion lens (n.a. = 1.4) and argon 488 nm and a He/Ne 543 nm lasers. The emission signals were filtered with a 515-565 nm filter (FITC) or with a long pass 595 nm
filter (TRITC). The images were recorded using identical laser power, wavelength and PM tube voltage. They were recorded digitally in a 768 x 556 pixel format and saved on a magneto optical disk.

*Preparation of cell extracts and immunoblotting*

Cell cultures were washed with PBS and lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mg/ml pepstatin, 1 mM leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM sodium pyrophosphate, and 100 mM sodium fluoride) containing, 2% (v/v) Triton X-100 and 1 µg/ml DNAse I. Lysates were cleared by centrifugation (10 000 g for 10 min) and both, supernatants (Triton-X100-soluble cytosolic fractions) and pellets, (Triton X-100-insoluble cytoskeletal fractions) were resuspended in electrophoresis sample buffer, heated at 100°C for 10 min and stored in aliquots at -20°C before processing.

Cell extracts were resolved on 10% polyacrylamide-SDS gels and electroblotted onto nitrocellulose membranes (Sartorius, Goettingen, Germany). Blots were processed using the "Western-Light Plus" chemiluminescent detection system (Tropix, Bedford, MA) according to manufacturers instructions. The following antibodies to MAP kinases were used: anti-p38 MAP kinase, anti-phospho-specific p38 MAP kinase (Thr180/Tyr182), anti-c-Jun, anti-phospho-specific c-Jun (Ser63) II, (New England Biolabs, Beverley, MA), anti-p46 JNK1 (C-17) (Santa Cruz, CA) and anti-active JNK pAb (Promega, Madison WI). Immunoreactive bands were visualized after exposure to Kodak BioMax Light-1 film (Eastman Kodak, Rochester, NY). In some cases, blots were stripped in buffer containing 200 mM glycine, 0.1% SDS, 1.0% Tween-20, pH 2.2, followed by washings before incubating with blocking buffer and with antibodies to
actin.
**Immunoprecipitation and assay of MAP kinases activities**

Cell lysates (400 µg protein) were incubated on a shaking platform overnight at 4°C with 5 µl of protein G-Sepharose conjugated with 1 µg of either anti-p38 MAP kinase or anti-p46 JNK1 antibodies (StressGen Biotechnologie Corp, Victoria, Canada). The immunoprecipitates were washed twice with buffer containing 20 mM HEPES, 10% glycerol, 0.1% Triton X-100 and 500 mM NaCl, followed by two washes with the same buffer containing 150 mM NaCl. Kinase activity was assayed by incubating the resuspended pellet at 30°C, in the presence of 10 µCi \( \gamma^32P \)ATP and the appropriate substrate: myelin basic protein (3 µg) for p38 MAP kinase and GST-c-jun (1µg) for JNK1. After 20 min, the reaction was stopped by the addition of electrophoresis sample buffer and heating at 100°C for 5 min. The proteins were resolved on 10% polyacrylamide-SDS gels and the gels were dried and exposed to Phosphoimager screens (BioMax Light-1, Kodak).

**Presentation of data**

Experiments were performed on at least six different cell cultures. In the figures, data are representative of a typical experiment and are given as the mean of at least three determinations (± S.E.). The comparison between data obtained under different experimental conditions was analyzed by Student's \( t \) test. Differences were considered to be statistically significant when \( P < 0.01 \).
RESULTS

**Neuronal death in neuron/microglial co-cultures exposed to chromogranin A exhibits apoptotic features**

We previously reported that exposure of neuron/microglial co-cultures to either bovine or recombinant human chromogranin A (CGA) induces a marked reduction in neuronal-specific \[^{3}H\]-GABA uptake and leads to neuronal degeneration (27). To further characterize neuronal death induced by CGA-activated microglia, we examined the binding of annexin V and the fragmentation of nuclear DNA in neuron/microglial co-cultures treated with 10 nM CGA. Binding of annexin V to the cell surface reveals the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer. When used in conjunction with propidium iodide exclusion to establish membrane integrity, it indicates cells undergoing apoptosis (34, 35). In addition, we used the TUNEL technique to label DNA fragments at the 3’-OH DNA, and thus identify nuclear DNA which is cleaved by endonucleases (35). Fig. 1 shows representative confocal images of neurons in co-culture with microglia for 3 days, incubated with or without 10 nM CGA. In untreated co-cultures, (Fig. 1A) annexin V (conjugated with fluorescein) did not bind to neurons, identified with anti-MAP2 antibody. In contrast, most neuronal bodies and processes in CGA-treated co-cultures were simultaneously stained for annexin V and MAP2 (Fig. 1B). In both untreated and CGA-treated co-cultures, nuclei were generally not labelled with propidium iodide (PI) when living cells were exposed to PI before fixation, indicating that the plasma membrane is impermeable to the dye. The percentages of PI-positive, MAP2-positive neurons in resting and CGA-stimulated co-cultures were $5.8 \pm 1.6$ and $9.5 \pm 1.9$ (mean ± S.E.; $n = 3$), respectively. Using the TUNEL technique, labelling of nuclei was observed in only a few
MAP2-positive neurons in untreated co-cultures (Fig. 1C). However, almost all nuclei of MAP2-positive neurons were stained in co-cultures exposed to CGA (Fig. 1D). In addition, CGA-treated co-cultures contained a substantial proportion of TUNEL-positive, MAP2-negative cells, probably corresponding to microglia. Taken together, these results suggest that CGA induces an apoptotic cascade leading to neuronal death in neuron/microglial co-cultures.

Chromogranin A-activated microglia secrete diffusible factors that induce neuronal apoptosis

To further analyze the neurotoxic effect of microglia activated by CGA, we performed experiments on neurons maintained in pure culture. We previously reported that the toxic factors secreted by CGA-activated microglia are released into the culture medium, and as such can be collected (27). Thus, cortical neurons were exposed to cell-free conditioned medium (CM) collected from either resting microglia or microglia treated for 24 h with 10 nM CGA, and the rate of apoptosis and necrosis was determined. In the early stages of apoptosis, the plasma membrane remains intact and impermeable to inert dyes such as trypan blue (36). In contrast, necrosis is accompanied by a rapid loss of membrane integrity and the cell membrane becomes permeable. As shown in Fig. 2, the proportion of necrotic neurons (stained with trypan blue) in CM from resting microglia does not exceed 10% of the total cell population. Furthermore, the percentage of apoptotic neurons revealed with the nuclear stain Hoechst 33342 remained lower than 15% over the whole experimental period. In contrast, in CM from CGA-treated microglia, the proportion of neurons with apoptotic Hoechst-positive nuclei rapidly increased and reached about 50% of the total neuronal population by 96 h. The number of neurons permeable to trypan blue also increased, but after a lag time of 48 h. These data indicate that the neurotoxic factors secreted by CGA-activated microglia do not directly affect the integrity of the neuronal
membrane, but are more likely to induce an apoptotic process in neurons.

**Effect of neurotoxic factors secreted by chromogranin A-treated microglia on MAP kinase phosphorylation and activity in neurons**

To investigate whether the neurotoxic factors secreted by CGA-treated microglia stimulate MAP kinases in neurons, we examined the presence and activation of c-Jun N-terminal kinase (JNK) and p38 MAP kinase, using antibodies which recognize the all forms, or the phosphorylated forms of the kinases. Fig. 3A shows that cultured cortical neurons express high levels of both JNK and p38 kinase. Both kinases were essentially present in the cytosol although a substantial fraction was also associated with cytoskeletal proteins. The relative distribution of total and phosphorylated forms of JNK was not significantly affected by the CM from resting or CGA-stimulated microglia. As expected, neurons also expressed c-Jun and its phosphorylated form (results not shown). In contrast, phosphorylated p38 kinase was not detected in neurons exposed to CM from resting microglia. However, exposure to CM from CGA-treated microglia triggered the rapid appearance of the phosphorylated form of p38 kinase, particularly in the cytosolic fraction (Fig. 3A).

To determine the time course of MAP kinase activation in response to CM from CGA-treated microglia, we measured kinase activities using appropriate substrates in immunoprecipitates obtained with specific antibodies. Fig. 3B shows that JNK is already activated in neurons and this activity does not significantly increase further upon exposure to CM from CGA-activated microglia. In contrast, CGA-induced microglial toxins provoked a marked and transient stimulation of p38 kinase in neurons. Note that preincubation with 10 μM SB 203580, a treatment reported to inactivate p38 MAP kinase in vitro (37), abolished the activation
of this enzyme in neurons (Fig. 3B).

To probe the involvement of p38 MAP kinase in the neurotoxic effects induced by CGA-stimulated microglia, we examined the ability of SB 203580 to prevent apoptosis in neurons. Fig. 3C shows that pretreatment of neuronal cultures with SB 203580 reduced, but did not completely prevented neuronal death induced by CGA-activated microglia. At 20 µM, SB 203580 protected approximately 60 % of the total neuronal population from microglial-induced apoptosis. These results provide evidence that p38 MAP kinase participates in the apoptotic cascade induced by CGA-stimulated microglia in neurons, although additional pathways are likely to be mobilized. Since activation of p38 kinase has been related to altered calcium homeostasis and/or production of free radicals, which are considered to be potent inducers of mitochondrial damage (2, 29), we examined whether the release of cytochrome c from mitochondria may account for the neuronal apoptosis induced by CGA-treated microglia.

Chromogranin A-stimulated microglia trigger the release of mitochondrial cytochrome c in neurons

The release of cytochrome c from mitochondria has recently been described as an early event that initiates apoptosis in neurons (2) and non-neuronal cells (29). Once released, cytochrome c interacts with Apaf-1 (apoptotic protease-activating factor 1) and caspase-9 to proteolytically activate procaspase-3. This activation of caspases is responsible for the morphological and nuclear changes associated with apoptosis (10, 38-40). To study whether microglial neurotoxins provoke the release of cytochrome c from mitochondria in neurons, we performed a double staining experiment with anti-cytochrome c antibody and the nuclear stain propidium iodide (Fig. 4). In neurons exposed to CM from resting microglia (Fig. 4A, B), anti-
cytochrome c antibody revealed a punctuate staining in cell bodies and processes, consistent with the mitochondrial location of this enzyme (40). This pattern of immunostaining was dramatically altered in neurons exposed to CM from CGA-stimulated microglia (Fig. 4C, D). After a 36 h exposure, the punctuate immunostaining observed with the anti-cytochrome c antibody had disappeared in most of the neurons, indicating the release of cytochrome c from mitochondria and its dilution into the cytoplasm. Time course analysis showed that release of mitochondrial cytochrome c occurred after 22 h of exposure to CGA-induced microglial toxins (Fig. 4E). At 36 h, more than 70% of the neurons in CM from CGA-stimulated microglia had lost their punctuate anti-cytochrome c staining and exhibit diffuse fluorescence with propidium iodide, indicating that the nuclear chromatin is not yet condensed (Fig. 4C, D). Thus, release of mitochondrial cytochrome c appears before development of nuclear apoptotic changes, suggesting that the leakage of cytochrome c from the mitochondria might account for the induction of neuronal apoptosis following treatment with medium from CGA-stimulated microglia.
Role of the Fas receptor in neuronal death induced by chromogranin A-stimulated microglia

The Fas/APO1/CD95 (Fas) transmembrane receptor including a cytoplasmic death domain (DD), and its ligand (FasL) have been recently described in neurons both in vitro (41, 42) and in the brain (43, 44). Ligation of Fas with soluble or membrane bound FasL activates DD, which in turn forms docking sites for the cytoplasmic adapter protein FADD/MORT1. FADD binds then to pro-caspase-8, thereby triggering its autocatalytic activation and the proteolytic cascade leading to cell death (28, 43, 45). FasL is also expressed on activated microglia and can be cleaved proteolytically from the cell surface by membrane-associated metalloproteinases (45-47). Therefore, we examined the possible participation of Fas/FasL in mediating neuronal apoptosis in response to CGA-activated microglia.

We first asked whether CGA may affect the expression or distribution of FasL in microglial cells. As illustrated in Fig. 5, immunostaining with polyclonal anti-FasL antibody revealed that FasL is already present in resting microglia (Fig. 5A). Treatment with CGA had no pronounced effect on the overall immunoreactivity but clearly induced the appearance of a thin slightly irregular staining highlighting the plasma membrane (Fig. 5B). Similar images were obtained with two distinct anti-FasL antibodies (results not shown). Thus, CGA seems to increase the association of FasL with the microglial cell surface.

To test the ability of Fas/FasL in triggering death of cultured cortical neurons, we used soluble FasL (sFasL), corresponding to a recombinant epitope-tagged form of the extracellular domain of human FasL. Incubation of Fas receptor-bearing cells with epitope-tagged sFasL and anti-tag antibodies, clusters sFasL and thereby Fas, leading to Fas activation (29). Addition of sFasL and anti-tag antibodies to neurons cultured in CM from resting microglia triggered apoptosis in a dose-dependent manner (Fig. 5C). The induction of neuronal apoptosis by sFasL
was also found in neurons maintained in unconditioned medium (results not shown). Note however, that sFasL (50 ng/ml) and CM from CGA-stimulated microglia had no additive effect on the percentage of apoptotic neurons, suggesting that the factors released by CGA-activated microglia and sFasL induce apoptosis through a common mechanism.

To further probe the involvement of Fas signalling in the induction of neuronal death by neurotoxins secreted by CGA-stimulated microglia, we assessed the effect of Fas-Fc, which contains the extracellular domain of recombinant human Fas fused to the Fc domain of IgG1, and antagonizes the activation of Fas by FasL (28, 42). Fas-Fc inhibited the death of neurons induced by a 48 h exposure to CM from CGA-stimulated microglia in a dose-dependent manner (Fig. 5D). The maximal effect (approximately 75 % inhibition) was observed at 5 µg/ml Fas-Fc, which is close to the dose which prevents the death of cultured motoneurons (42). Signalling through Fas-receptors involves activations of caspase-8 followed by the downstream caspases, including caspase-3. Caspase-3 is also activated by caspase-9 in response to the release of mitochondrial cytochrome c (29, 48). To test the involvement of these caspases in the death process induced by neurotoxins from CGA-stimulated microglia, we used the cell-permeable peptides IETD and DEVD which are reported to inhibit irreversibly and selectively caspase-8 and caspase-3, respectively (49). Addition of these inhibitors to cultures of neurons, 1 h before incubation in CM from CGA-stimulated microglia, prevented neuronal death (Table 1). After 48 h, IETD and DEVD reduced the apoptotic effect of CGA-activated microglia by 67 % and 80 %, respectively, consistent with the requirement for caspases-8 and -3 in CGA-mediated neuronal apoptosis.
DISCUSSION

Microglia are the resident macrophages of the central nervous system. They play an important role in determining neuronal survival and differentiation, and provide the nervous system with a line of defense against damage and infection by killing invading microorganisms and removing dying cells. Thus, the activation of microglia represents a beneficial physiological response in host defense. However, sustained microglial activity may contribute to the pathogenesis of neurodegeneration through the extensive release of various neurotoxic agents, such as proteases, proinflammatory cytokines, and reactive oxygen and nitrogen intermediates (3-7). The specific molecules that control microglia activity within the brain remain elusive. In numerous neurodegenerative diseases, CGA is up-regulated and colocalizes with reactive microglia. CGA is particularly prominent in senile plaques and constitutes one of the major protein along with β-amyloid protein in extracellular deposits associated with Alzheimer's disease (11, 15-17). The presence of CGA in brain lesions led us to examine whether CGA could contribute to neuronal degeneration. We found that CGA can induce an activated phenotype in rodent microglial cells (26) and triggers the release of microglial factors that cause injury and degeneration of brain cortical neurons (27).

In the present study, we examined the mechanisms by which neurotoxins released from microglia in response to CGA provoke neuronal death. Treatment of neuron/microglial co-cultures with CGA triggers two features characteristic of apoptotic death (1, 36): the translocation of phosphatidylserine from the inner to the outer face of the plasma membrane and intranucleosomal cleavage of DNA in neurons. Culture medium conditioned by CGA-activated microglia induces apoptosis in neurons after a delay of 22 hours, an observation which is
consistent with the release of diffusible neurotoxins from CGA-treated microglia and the subsequent activation of a death program in neurons. Thus, we propose that CGA represents an endogenous brain factor that drives microglia to a neurotoxic phenotype.

Numerous studies have implicated MAP kinase pathways in the induction of neuronal death, although their precise role is still unclear (1). JNK activation and phosphorylation of c-Jun have been involved in the induction of apoptotic neuronal cascades in Alzheimer’s disease (10, 11), and JNK has been associated with apoptosis induced by HIV-1 in human neurons (50). However, c-Jun expression has also been linked to axonal regeneration and neuronal survival (51). We show here that exposure of neurons to CGA-induced microglial neurotoxins does not significantly modify JNK activity, but induces a marked and transient activation of p38 MAP kinase, which preceded the onset of neuronal apoptosis. Pretreatment of neurons with a selective inhibitor of p38 MAP kinase partially protected neurons, arguing that p38 kinase is a possible signal transducer for neuronal apoptosis induced by CGA-activated microglial neurotoxins. In cerebellar granular neurons, activation of p38 MAP kinase has been linked with apoptosis induced by NMDA glutamate receptor (52). In this context, it is interesting to note that Kingham et al. (1999) described the release of glutamate from cultured microglia stimulated with CGA (7). The activation of p38 kinase has been related to altered calcium homeostasis and increase in free radicals and reactive oxygen species (2), which are considered as potent inducers of mitochondrial damage (2, 29). Indeed, we found that exposure of neurons to CM from CGA-activated microglia triggers the release of mitochondrial cytochrome c into the cytosol prior to the development of nuclear condensation. Within the cytosol, cytochrome c is one of the cofactors that promotes caspase-9 activation, which in turn mobilizes downstream caspases such as caspase-3 (29). Taking the observation that cytochrome c leakage from mitochondria preceded
the appearance of apoptosis together with the neuronal protection obtained with a caspase-3 inhibitor, further support the participation of the p38 MAP kinase/cytochrome c-activated cascade in the induction of neuronal apoptosis.

Although few data exist on the involvement of FasL/Fas in neurodegenerative diseases, FasL and Fas have been recently identified on neurons in vitro and in the brain (41-44). In addition, Fas was detected in adult postmortem brains of patients with neurodegenerative disorders, including Alzheimer's disease (53). As an indication of the role of FasL/Fas signalling in the induction of neuronal death by microglial neurotoxins, we found that Fas-Fc, a known antagonist of Fas activation by FasL (28, 42), protected neurons exposed to CGA-induced microglial neurotoxins. Microglia express FasL and upon exposure to CGA, FasL is more prominent on the plasma membrane. These findings are in line with previously published data describing the constitutive expression of FasL by microglia in culture and in brain, and its up-regulation when microglia are activated (47). Since membrane-associated FasL can be cleaved by metalloproteinase to generate the active soluble form of the ligand (45, 46), soluble FasL from CGA-stimulated microglia might contribute to the induction of apoptosis in neurons. Accordingly, recombinant soluble FasL triggered apoptosis in neurons in a dose-dependent fashion, and neuronal death induced by microglial neurotoxins could be prevented by cell-permeable peptides that inhibit caspase-8 and -3 involved in FasL/Fas signalling. Thus FasL of microglial origin may well by one of the neurotoxins secreted by microglia in response to CGA activation.

In conclusion, we provide evidence that neurotoxins released from microglia activated by CGA, trigger apoptotic death of cortical neurons through pathways involving FasL/Fas receptor and p38 MAP stress kinase as signal transductors, and mitochondrial cytochrome c and caspases-
8 and-3 as apoptotic mediators. The neurotoxicity of activated microglia can be attributed to a variety of secretory products, including cytokines, complement proteins, proteolytic enzymes, reactive oxygen intermediates and nitric oxide, NMDA-like toxins (3-7, 26, 27, 54) and, as shown here, membrane-bound or soluble FasL. Thus a combination of these factors probably mobilizes several death-signalling pathways in target neurons. The present findings together with the recent reports describing the toxic cascades induced by CGA in microglial cells (7, 54) establish CGA as a novel endogenous activator of microglia able to induce inflammatory processes in the brain. Inflammatory microglia are characteristic of many neurological diseases and the identification of the responsible agents may therefore be relevant for novel therapeutic strategies. Increased levels of CGA in cerebrospinal fluids have been found in patients with Parkinson disease (55) and has been correlated with early synaptic degeneration in Alzheimer's disease (56). Our findings point to the possibility that CGA accumulation is an early marker for the diagnosis of brain inflammation and that CGA-induced neurotoxicity may be limited by the use of anti-inflammatory drugs.

ACKNOWLEDGEMENTS We thank Dr. N.J. Grant and Dr. K. Langley for suggestions and revising the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1: Neuronal death in neuron/microglial co-cultures exposed to chromogranin A. Neuron/microglial co-cultures were either untreated (A, C) or treated for 3 days with 10 nM CGA (B, D). In A, B living cells were incubated with propidium iodide (2.5 µg/ml) in culture medium and then with fluorescein-conjugated annexin V. After fixation, cells were labelled with anti-MAP2 antibody revealed with rhodamine. In C, D fixed cultures were processed with TUNEL technique using dUTP-biotin and streptavidin-fluorescein and with anti-MAP2 antibody revealed with rhodamine. Confocal images obtained in the rhodamine and fluorescein channels were recorded simultaneously in the same optical section by a double exposure procedure. The arrow points to a TUNEL-positive MAP2-positive neuron. The arrowhead indicates several TUNEL-positive, MAP2-negative cells with smaller nuclei which are presumed to be microglia. Bar = 25 µm.

Figure 2: Neurotoxic factors released from chromogranin A-activated microglia induce apoptosis in neurons. Cultured neurons were grown in cell-free medium collected either from resting microglia (open symbol) or from microglia stimulated with 10 nM CGA (closed symbol) and necrotic and apoptotic neurons were counted. Living neurons labelled with trypan blue (0.1%, 5 min incubation) were considered as necrotic. The presence of apoptotic profiles was revealed in neurons after fixation, by staining with Hoechst 33342 dye (2.5 µg/ml, 5 min). Neurons with segmented and highly condensed nuclear fluorescence were counted as apoptotic and expressed as percentage of the total number of cells present per field. Values are the means of eight determinations ± S.E. and are representative of at least three independent experiments.
Figure 3: Effect of conditioned medium from resting or chromogranin A-stimulated microglia on JNK and p38 MAPK in neurons. (A) Western blotting showing the protein level of the total and phosphorylated forms of JNK and p38 MAP kinases in neurons incubated for 15 min with medium from microglia cultured without (- CGA) or with 10 nM CGA (+ CGA). Triton X-100-soluble cytosolic (1) and Triton X-100-insoluble cytoskeletal (2) protein fractions from neurons were resolved by electrophoresis, transferred onto nitrocellulose and blots were probed with antibodies against p46 JNK1 (JNK), phosphorylated JNK (pJNK), p38 MAP kinase (p38) and phosphorylated p38 MAP kinase (pp38). Immunodetection of actin (arrowhead) is shown as an internal standard for protein loading. The results shown are representative of four independent experiments. (B) The activity of JNK or p38 MAPK was measured in immunoprecipitates from neurons exposed for the indicated period of time to CM from resting (- CGA) or CGA-stimulated (+ CGA) microglia. To inhibit p38 MAPK, neurons were incubated 1 h with 30 µM SB 203580 prior to addition of CM. The radioactivity incorporated into the substrates was estimated after electrophoresis by optical scanning densitometry on the corresponding autoradiogram. Values are expressed relative to the kinase activities detected in neurons incubated for 15 min with CM from resting microglia. Results are representative of three independent experiments. Student's t test was used for estimating significance: * * P < 0.001. (C) Neurons were incubated for 48 h with CM from resting (- CGA) or CGA-stimulated (+ CGA) microglia in presence of the indicated concentrations of SB 203580 added 1 h prior to treatment with CM. The number of apoptotic nuclei was assessed by Hoechst 33342 staining. Data are the mean percentage of apoptotic cells ± S.E. and are representative of three independent experiments. t test, * P < 0.01.
**Figure 4:** Neurotoxic factors secreted by chromogranin A-activated microglia induce the release of mitochondrial cytochrome c in neurons. Representative photographs of fixed cultured neurons double-labelled with anti-cytochrome c antibody (A, C) and the nuclear stain propidium iodide (B, D). Neurons were exposed for 36 h to CM collected from resting microglia (A, B) or to CM from CGA-activated microglia (C, D). Confocal images were taken under identical set-up conditions. Bar = 12 µm. The arrow points to a neuron with reduced immunoreactivity for cytochrome c (C) and having a nucleus showing non-condensed chromatin (D). (E) Time course of the loss of anti-cytochrome c immunostaining in neurons exposed to CM from CGA-stimulated microglia. Values are means of eight determinations performed with the three cultures ± S.E.

**Figure 5:** FasL/Fas signalling in neuronal apoptosis triggered by CGA-activated microglia.
Double-staining with anti-FasL antibody and propidium iodide (2.5 µg/ml) of microglial cells untreated (A) and treated with 10 nM CGA (B). Note the appearance of FasL immunoreactivity on the plasma membrane of microglia exposed to CGA (arrows). Bar = 12 µm. (C) Effect of soluble FasL (sFasL) on neuronal apoptosis. Tagged sFasL at the indicated concentrations together with 1 µg/ml of anti-tag antibody were added to neurons cultured with CM from resting (- CGA) or CGA-stimulated microglia (+ CGA). Apoptotic nuclei were assessed by Hoechst 33342 staining after 24 h. *t* test, * P > 0.01. (D) Effect of Fas-Fc, an inhibitor of Fas activation by FasL, on neuronal apoptosis induced by CGA-induced microglial toxins. Neurons were preincubated for 30 min with the indicated concentrations of Fas-Fc and enhancer antibody (1 µg/ml) before addition of CM from resting (- CGA) or CGA-stimulated (+ CGA) microglia. Apoptotic cells were revealed after 48 h by Hoechst staining. Histograms are representative of
three independent experiments. Data are the mean percentage of apoptotic cells ± S.E.
Table 1
Death of cortical neurons triggered by neurotoxic factors from CGA-activated microglia involves caspases-8 and -3.

<table>
<thead>
<tr>
<th></th>
<th>% of apoptotic neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13 ± 2.8</td>
</tr>
<tr>
<td>+ CGA</td>
<td>30 ± 4.6</td>
</tr>
<tr>
<td>+ CGA + IETD</td>
<td>18 ± 3.0</td>
</tr>
<tr>
<td>+ CGA + DEVD</td>
<td>16 ± 2.0</td>
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</tbody>
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IETD (10 µM), a caspase-8 inhibitor, and DEVD (50 µM), an inhibitor of caspase-3, were added to cultures of neurons 1 h before incubation with CM from CGA-activated microglia (+ CGA). Apoptotic nuclei were assessed by Hoechst 33342 staining after 48 h of incubation. Data are the mean percentage of apoptotic cells ± S.E. and are representative of three independent experiments.
Mechanisms underlying neuronal death induced by chromogranin A-activated microglia
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J. Biol. Chem. published online December 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M009711200

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