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Complement-Derived Anaphylatoxin C5a Protects Against Glutamate-Mediated Neurotoxicity

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Abstract Previous work from this laboratory indicates a role for the complement component C5 in neuroprotection against excitotoxicity. In the present study, we tested the hypothesis that the C5-derived anaphylatoxin C5a protects against kainic acid (KA)-induced neurodegeneration and investigated the mechanism of C5a neuronal activity in vitro. Brain intraventricular infusion of KA into adult mice caused neuronal morphological features of apoptosis in the pyramidal layer of the hippocampal formation as indicated by counts of neurons with pyknotic/condensed nuclei associated with cytoplasmic eosinophilia. Co-intraventricular infusion of human recombinant C5a with KA resulted in a marked reduction of morphological features of apoptotic neuronal death. In vitro studies confirmed C5a neuroprotection: treatment of primary murine corticohippocampal neurons with human or mouse recombinant C5a reduced glutamate neurotoxicity, as measured by trypan blue exclusion assay. This protection concurred with inhibition of glutamate-mediated induction of the caspase-3-related cysteine protease and coincided with marked reduction of neurons with morphological features of apoptosis, as found in vivo. Our studies indicate that C5a may inhibit glutamate-mediated neuronal death through partial inhibition of caspase-3 activity. These findings suggest a novel noninflammatory role for C5a in modulating neuronal responses to excitotoxins. J. Cell. Biochem. 73:303–311, 1999. © 1999 Wiley-Liss, Inc.

Key words: complement; caspase; Alzheimer's disease; inflammation; anaphylatoxin

The complement system, a major component of the inflammatory response, plays a significant role in neurodegeneration [Akiyama et al., 1991; McGeer et al., 1991, 1996] and contributes to the pathophysiology of Alzheimer's disease (AD) [McGeer et al., 1989; Pasinetti, 1996; Shen et al., 1997; Walker and McGeer, 1992; Rogers et al., 1996]. We have used mice with genetically induced C5 deficiency to study the role of this complement protein in neurodegenerative mechanisms. Surprisingly, C5-deficient mice show more neuronal injury to excitotoxicity, an effect that may be mediated by changes in calcium-dependent alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor binding [Pasinetti et al., 1996; Tocco et al., 1997]. These studies suggest that in addition to their contribution to neurodegenerative mechanisms, complement proteins may be neuroprotective.

During complement activation, anaphylatoxins are released and interact with cellular components to amplify inflammatory processes. One such peptide is C5a, a glycoprotein cleaved from C5 by C5 convertase [Cochran and Müller-Eberhard, 1968; Gerard and Gerard, 1994; Hugli, 1984]. In vitro, C5a is chemotactic for astrocytes and brain-derived macrophage/microglia [Yao et al., 1990]. The response of inflammatory cells to C5a is mediated by binding to the C5a receptor (C5aR) [Gerard and Gerard, 1994, 1991; Chenoweth and Hugli, 1978; Höpken et al., 1996]. We and others have found that C5aR is expressed in the neurons and glia of mouse brain [Osaka et al., 1998; Stahel et al., 1997], where it is regulated in response to kainic acid (KA) excitotoxic lesions [Osaka et al., 1998]. This finding suggests that the influence of C5 on the response to excitotoxic insults could be mediated by binding of C5a to its neuronal receptor. In this study, we examined the neuro-

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protective role of C5a in excitotoxicity both in vivo and in vitro.

MATERIALS AND METHODS

Anaphylatoxins

Purity of the human recombinant (hr)C5a (Sigma Chemical Co., St. Louis, MO) and murine recombinant (mr)C5a (gift of Dr. Scott Barnum) was verified by polyacrylamide gel electrophoresis (PAGE)-Coomassie blue (BRL) staining (see Results). All cultures and reagents were demonstrated to be free of endotoxin (<10 pg/ml) by *Limulus* lysate assay (Sigma, St. Louis, MO).

Chemokinetic potency of hrC5a (EC_{50}: 1.2×10^{-10} M in human neutrophil) was assessed for biological activity. In neuronal SH-SY5Y cells, hrC5a demonstrated the ability to induce protein kinase Cα activity and mobilize Ca^{2+} in individual cells by fura-2 radiometric imaging (H. Osaka and G.M. Pasinetti, unpublished observations). Biological activity of hrC5a was also confirmed in astrocytes; hrC5a induction of mitogen-activated protein kinase (ERK1) was receptor mediated, as no activation was found in astrocytes derived from C5a receptor knockout mice [Höpken et al., 1996; Osaka et al., 1998].

Kainic Acid (KA) Lesions

For KA neurodegeneration studies, adult B10.D2/nSnj mice (Jackson Laboratories, Bar Harbor, ME) were used. KA lesions were obtained by intraventricular (ICV, lateral ventricle) infusion of KA (4.6 nmoles) and/or hrC5a (Sigma, 4.8 μM) in 2 μl vol, as previously described [Osaka et al., 1998]. The dose of hrC5a for ICV infusion was selected to achieve a cerebrospinal fluid (CSF) concentration similar to the effective hrC5a concentration (100 nM) in the in vitro studies below. Mice were monitored for seizure activity; all the injected mice exhibited an apparent homogeneity of seizure activity [Tocco et al., 1997]. Neuronal features of apoptotic death were assessed by counting neurons with evident pyknotic condensed nuclei surrounded by cytoplasmic eosinophilia, using H&E histochemistry as previously described [Tocco et al., 1997].

Primary Neuron Cultures

Primary cortico-hippocampal cultures of mouse embryonic neurons (gestational day 14–16) were prepared as previously described [Pasinetti et al., 1996] with minor modifications. Neurons were seeded at 2×10^5 per well in poly-D-lysine (Sigma) coated 96-well plates and cultured in serum-free chemically defined medium Neurobasal/B27 (2%) supplement and 1% Penicillin-Streptomycin (Gibco-BRL, Gaithersburg, MD). The absence of astrocytes (<1–2%) was confirmed by the lack of glial fibrillary acidic protein (GFAP) immunostaining verified in parallel studies (data not shown). Northern blot hybridization of total RNA was used to verify C5a receptor expression in these primary corticohippocampal neurons as previously described [Osaka et al., 1998].

Determination of Glutamate Neurotoxicity

For glutamate neurotoxicity, 7- to 8-day-old cultures of primary mouse cortico-hippocampal neurons were exposed to l-glutamate (Sigma) for the appropriate time and dose. Neurotoxicity was determined by the quantification of trypan blue (Sigma)-positive neurons in 8–10 randomly selected fields. Assessment of morphological features of apoptotic damage was done by counting neurons with evident pyknotic condensed nuclei surrounded by cytoplasmic eosinophilia using H&E histochemistry as previously described [Tocco et al., 1997]. Damaged neurons were quantified from 8–10 randomly selected fields. Six independent lots of hrC5a from Sigma were used in these studies; all lots exhibited consistency for purity, biological activity, and neuroprotection in primary neuronal cultures.

Caspase-3 Protease Activity

Treated primary cortico-hippocampal neuron cultures were washed with phosphate-buffered saline (PBS) and harvested in 100 μl cell lysis buffer (50 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 0.5 mg/ml PEFA block-SC). Lysates were then incubated on ice for 15 min and centrifuged at 10,000*g* for 10 min at 4°C, and protein concentrations were determined. Lysates (soluble supernatants) were used immediately or stored at −80°C. Aliquots of protein (50 μg) were incubated at 37°C with assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and 200 mM Ac-DEVD-pNA (Biomol, Plymouth Meeting, PA). Caspase-3-like activity was monitored colorimetrically (405 nm) by quantifiying the release p-nitroaniline

from the cleaved caspase-3 substrate DEVD-pNA at appropriate time intervals. The addition of the caspase-3 inhibitor Ac-DEVD-CHO (0.1 μM) to reaction mixture blocked the glutamate induced caspase-3-like activity, confirming specificity of the caspase-3 assay.

Statistical Analysis

Analysis of variance (ANOVA) was used to compare three or more treatments. Bonferroni's multiple comparison test was used to detect differences between treatments.

RESULTS

hrC5a Protects Against Hippocampal KA Neurodegeneration

To evaluate the role of hrC5a on hippocampal KA neurodegeneration we counted visibly damaged neurons in mouse brain after KA lesions. By 72 h after ICV KA infusion (4.6 nmoles in 2 μl), we observed an increased number of damaged neurons with morphological features of apoptosis characterized by condensed pyknotic nuclear morphology associated with cytoplasmic eosinophilia. Changes were most evident in the CA3 pyramidal layer of hippocampal formation ipsilateral to the lesion compared with saline-injected controls (Figs. 1A, 2A,B). Co-ICV infusion of KA with hrC5a (final concentration of infused hrC5a: 4.8 μM) coincided with a marked reduction in the number of damaged neurons at 72 h postlesioning (Figs. 1A, 2C vs 2B). Protection was also observed in the CA1 subdivision of the pyramidal layer and in the temporal/cingulate cortex ipsilateral to injection side (data not shown). No detectable glutamate-mediated neuronal damage was found in the granule cell layer of the dentate gyrus (Figs. 1B, 2D–F). ICV infusion of hrC5a alone had no effect on basal level of neuronal survival in any hippocampal region examined (Fig. 1A,B).

hrC5a Protects Against Glutamate Neurotoxicity In Vitro

Exposure of primary cortico-hippocampal neurons to glutamate (25–50 μM) resulted in concentration- and time-dependent loss of neuronal viability as assessed by vital dye trypan blue assay (Fig. 3A,B). Neurotoxicity was assessed by quantification of trypan blue-positive neurons counted in 8–10 randomly selected

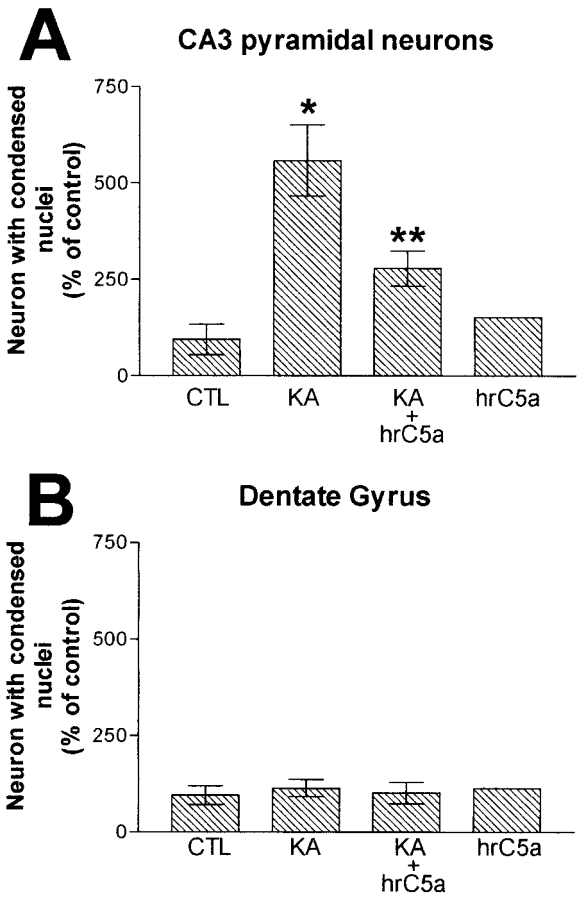


Fig. 1. hrC5a protects against hippocampal KA neurodegeneration hematoxylin and eosin (H&E) histochemistry was used to assess morphological features of apoptotic neuronal death in **(A)** CA3 subdivision of the pyramidal layer and **(B)** granule cell layer of dentate gyrus of the mouse hippocampal formation. For each animal, 5–6 rostrocaudal tissue sections encompassing the dorsal hippocampal formation were used for estimation of damaged neurons characterized by condensed, pyknotic nuclear morphology associated with cytoplasmic eosinophilia. Values are mean ±SEM; n = 3–4 per group, hrC5a n = 1; *$P < 0.01$ vs control value and **$P < 0.05$ compared with KA lesion group. CTL, control; KA, kainic acid; hrC5a, human recombinant C5a.

fields per group from two to three independent studies.

To assess the role of C5a (hrC5a) in neuroprotection, neuronal cultures were pretreated for 24 h with either hrC5a or mrC5a and then exposed to glutamate; neurotoxicity was assessed by quantification of trypan blue-positive neurons from four to five independent studies. Neurotoxicity induced by exposure of cultures to 50 μM glutamate for 24 h (Figs. 3C, 4C) was significantly reduced by pretreatment with 100 nM of either hrC5a or mrC5a (Figs. 3C, 4D vs 4C). Neuroprotection by hrC5a was dose depen-

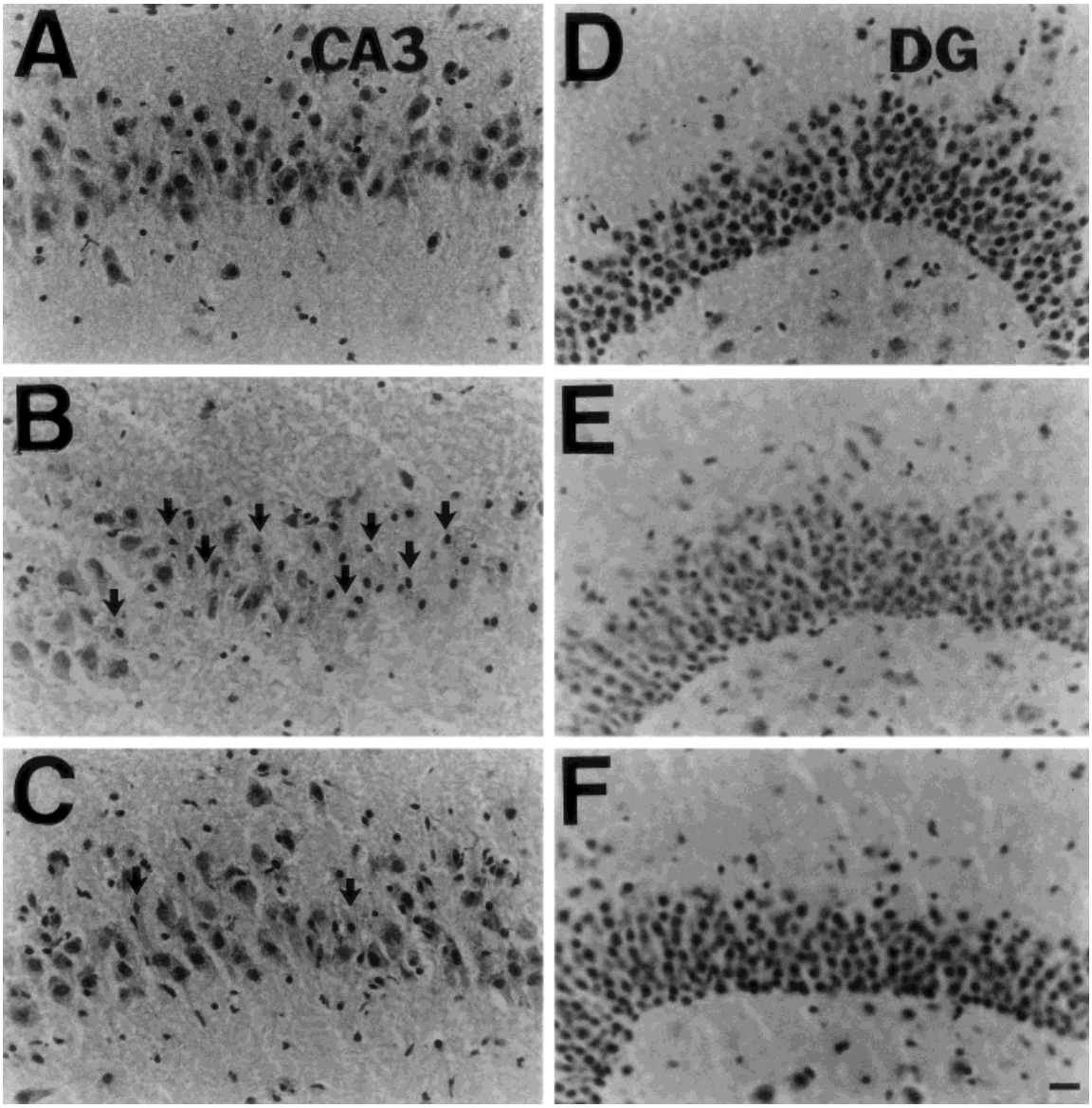


Fig. 2. hrC5a protects against hippocampal KA neurodegeneration. Micrographs of the CA3 subdivision of the pyramidal layer (A–C), and granule cell layer of the dentate gyrus (DG) (D–F), from mouse brain tissue sections visualized by hematoxylin and eosin (H&E) histochemistry. **A,D:** Control vehicle-injected group. **B,E:** Glutamate-treated. **C,F:** Glutamate/hrC5a-treated. Scale bar = 30 μm.

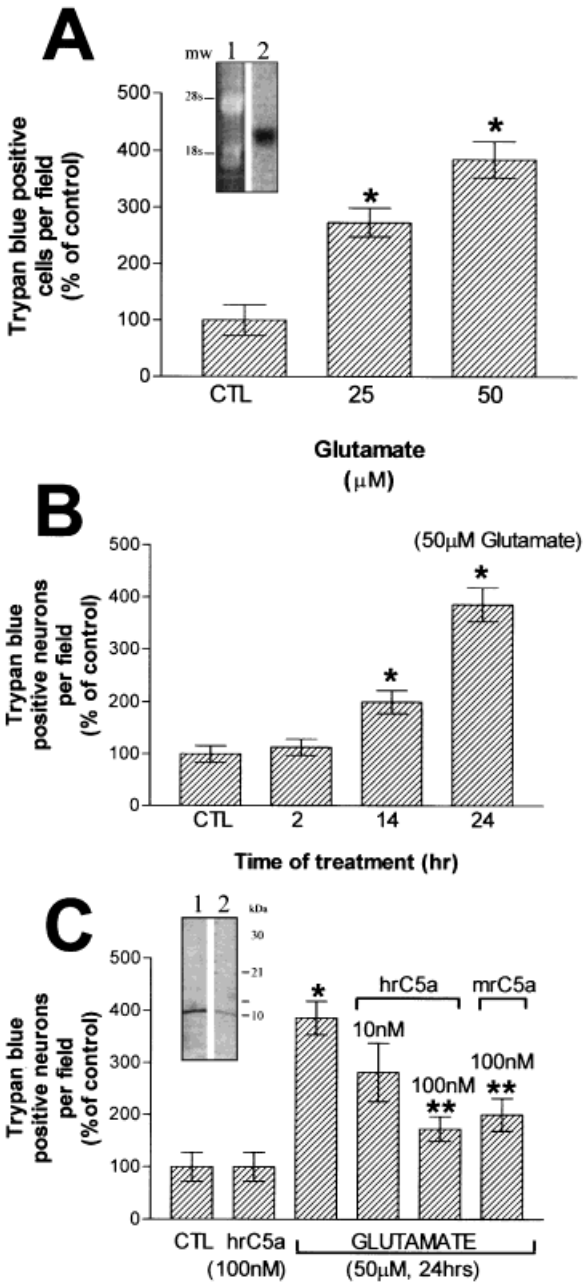
dent and was not detected in neuronal cultures pretreated with 10 nM hrC5a (Fig. 3C). Cultures treated with hrC5a for 24 h did not differ from control cultures (Figs. 3C, 4A,B).

Northern blot hybridization of total RNA (30 μg) from primary cortico-hippocampal neurons (8-day-old cultures) was used to assess C5aR mRNA expression in primary cortico-hippocampal neurons using a mouse specific [^{32}P]-C5aR cRNA probe. Hybridization revealed a single mRNA species with a molecular weight slightly greater than 18S RNA (approximately 2.2 kb) (Fig. 3A, inset). PAGE/Coomassie blue staining confirmed purity of hrC5a and mrC5a and displayed a unique band of approximately 9–10 kDa, as expected (Fig. 3C, inset)

Glutamate-Mediated Activation of Caspase-3 in Cortico-Hippocampal Neurons

To assess the role of glutamate in activation of caspase-3-like protease activity in primary corticohippocampal neurons, we monitored the release of p-nitroaniline from the caspase-3-specific substrate Ac-DEVD-pNA from lysates

of glutamate-treated cortico-hippocampal neuron cultures as assessed by colorimetric assay (Fig. 5A). We found that caspase-3-like protease activity in glutamate-treated neurons was linear over a range of time (Fig. 5A) and protein concentration (not shown). Caspase-3-like protease activity in lysates of glutamate-treated neurons was inhibited by the addition of the caspase-3 tetrapeptide specific inhibitor Ac-DEVD-CHO to the lysate-substrate reaction mixture (Fig. 5A).



hrC5a Inhibits Glutamate-Mediated Induction of Caspase-3-Like Activity in Cortico-Hippocampal Neurons

Caspase-3-like protease activity in lysate extracts of glutamate-treated cortico-hippocampal neuron cultures revealed four- to fivefold elevation by 24 h after treatment with 50 μM glutamate, as compared with untreated control neuron cultures (Fig. 5B). Neuron cultures pretreated with hrC5a for 24 h before glutamate exposure showed marked reduction of caspase-3-like protease activity in response to glutamate treatment (Fig. 5B). Neuronal cultures treated with hrC5a for 24 h did not differ from control cultures (Fig. 5B).

The neuroprotective role of hrC5a against glutamate neurotoxicity in cortico-hippocampal cultures was further assessed by changes in nuclear morphology and cellular integrity using H&E histochemistry (Fig. 5B panels 1–4, inset). By 24 h after exposure to glutamate (50 μM), we found greater than threefold elevation in number of cortico-hippocampal neurons with morphological features of apoptotic damage which coincided temporally with the induction of caspase-3-like activity (see above). Neuronal damage was characterized by condensed pyknotic nuclear morphology associated with cytoplasmic eosinophilia (Fig. 5B, panel 3), as compared with control untreated cultures (Fig. 5B, panel 1). Neuron cultures pretreated for 24 h with hrC5a (100 nM) showed a marked reduction in the number of damaged neurons coinci-

Fig. 3. hrC5a and mrC5a protect against glutamate-mediated neurotoxicity. **A:** Dose curve. **B:** Time course of glutamate-mediated neurotoxicity, as assessed by vital dye trypan blue exclusion assay. **C:** Neuronal cultures were pretreated with hrC5a or mrC5a for 24 h and then exposed to different concentrations of glutamate; neuronal survival was assessed 24 h later. Trypan blue solution (Sigma, 0.02% in culture medium) was added to cultures for 20 min. Cultures were rinsed in phosphate-buffered saline, and trypan blue-positive cells were counted per low magnification field, as indicated in Materials and Methods. Neurotoxicity was determined by the quantification of trypan blue-positive neurons, which were counted in 8–10 randomly selected fields (premarked reticules of 1 mm^2). Values represent means ±SEM of determinations made in 4–5 separate cultures; $*P < 0.001$, compared with control value in cultures exposed to glutamate and $**P < 0.01$, compared with corresponding values for cultures treated with glutamate. A (inset): Northern blot hybridization of total RNA showing C5aR mRNA expression in parallel cultures to those used for glutamate neurotoxicity. **Lane 1**, ethidium bromide staining of total RNA; **lane 2**, C5aR mRNA hybridization signal. C (inset): PAGE/Coomassie blue staining confirmed purity of hrC5a (**lane 1**) and mrC5a (**lane 2**), showing a unique band of approximately 9–10 kDa, as expected.

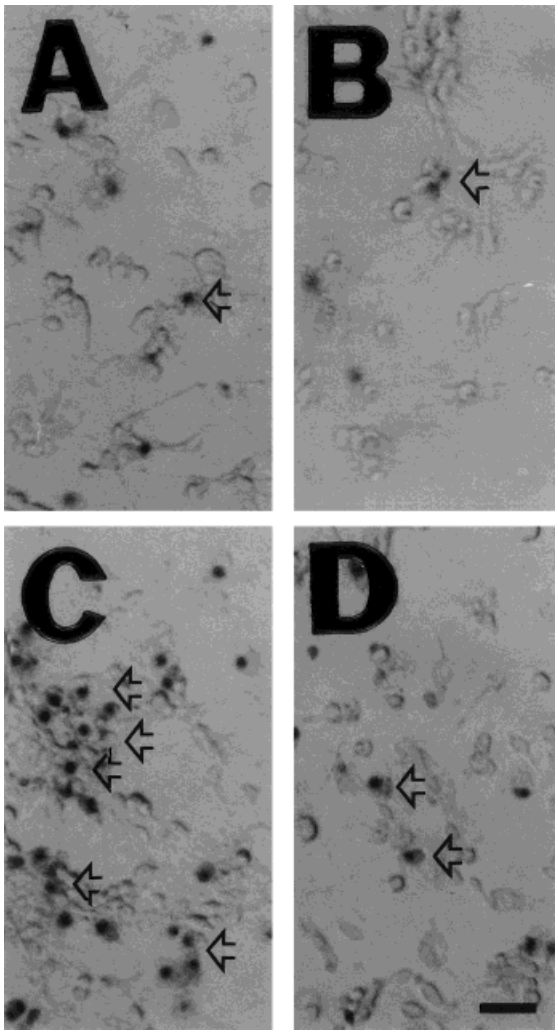


Fig. 4. hrC5a protects against glutamate neurotoxicity as assessed by trypan blue assay. **A:** Micrograph of control-untreated corticohippocampal neuron cultures. **B:** Cultures treated with hrC5a alone for 24 h. **C:** Loss of neuronal viability is visualized by an increase in the number of trypan blue-positive neurons 24 h after exposure to 50 μM glutamate. **D:** Glutamate neurotoxicity was significantly reduced by pretreatment with 100 nM of hrC5a for 24 h. C,D: Large arrows, trypan blue-positive neurons. Arrows, undamaged neurons. Scale bar = 30 μm.

dental to inhibition of caspase-3-like activity (Fig. 5B, panel 4 vs panel 3). Neuronal cultures treated with hrC5a for 24 h did not differ from control cultures (Fig. 5B, panel 1 vs panel 2).

DISCUSSION

Our data suggest that hrC5a protects against glutamate-mediated neurodegeneration in vivo and in vitro. Co-intraventricular infusion of hrC5a with KA markedly reduced the number of neurons with morphological features of apoptotic damage in the hippocampal formation. Further, treatment of cortico-hippocampal cultures with hrC5a and mrC5a resulted in substantial protection against glutamate toxicity.

Little is known about the role of C5a in the normal and pathological brain. In this study, we found that 100 nM hrC5a is an effective neuroprotective concentration with regard to glutamate neurotoxicity. We note that a similar range of human (h)C5a concentrations (60–80 nM) are required to induce interleukin-8 (IL-8) secretion in human monocytes [Ember et al., 1994]. Therefore, despite the different source of C5a, cell type, and assay system, the C5a doses required for cytokine secretion (inflammation?) and neuroprotection are remarkably similar.

We recently found that C5a receptor (C5aR) is expressed in neuronal (and glial) cells of the mouse brain [Osaka et al., 1998]. Moreover, using ligand binding autoradiography, we found that C5aR is dynamically regulated in mouse brain during responses to KA lesions [Osaka et al., 1998]. Therefore, the level of C5aR expression may also play an important role in the amplification of C5a-mediated responses during neurodegeneration, as found in the present study.

We found that hrC5a neuroprotection coincided with marked inhibition of the activity of the cysteine protease caspase-3. Caspase-3, a member of the interleukin-1β (IL-1β) converting enzyme-like protease family [Alnemri et al., 1996], has been shown to mediate apoptotic death in many cell types and conditions [Janicke et al., 1998; Cohen, 1997; Yakovlev et al., 1997], including primary neuronal cultures after glutamate treatment [Du et al., 1997]. Coincidental apoptotic neuron death and caspase-3-like activity was demonstrated in AD brain [Yang et al., 1998]. While controversial, caspase-3 activity has also been linked to the proteolytic processing of presenilin-1 and presenilin-2, which may be important to the pathophysiology of AD [Kim et al., 1997; Grunberg et al., 1998].

Previous studies found that relatively low concentrations of glutamate treatment leads to induction of caspase-3 coincidental to apoptotic neuronal death [Du et al., 1997]. Under similar experimental conditions, we confirmed these findings in corticohippocampal neurons and found that C5a anaphylatoxin may be neuroprotective through the regulation of caspase-3 activity. However, our studies do not provide specific information on the mechanism by which

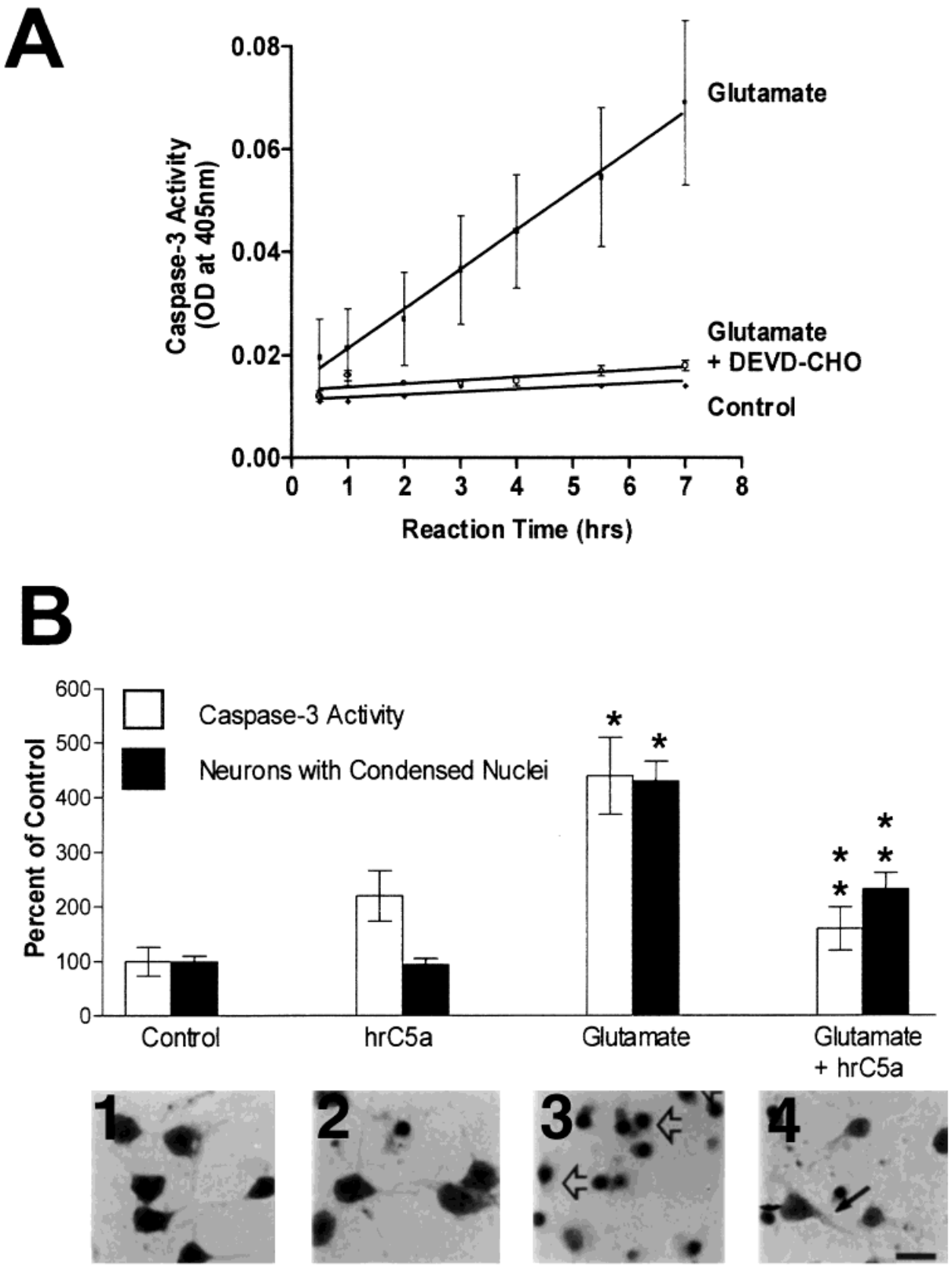


Fig. 5. Inhibition of caspase-3 activity by hrC5a coincides with protection against glutamate neurotoxicity as assessed by H&E histochemistry. **A:** Caspase-3 like protease activity in lysate extracts of glutamate-treated cortico-hippocampal neuron cultures (24 h after treatment with 50 μM glutamate) and inhibition of the glutamate-mediated induction of caspase-3 like activity by tetrapeptide-specific inhibitor Ac-DEVD-CHO. **B:** Pretreatment of cortico-hippocampal cultures for 24 h with hrC5a (100 nM) showed marked reduction of caspase-3 like protease activity coincidental with reduction of neurons with morphological features of apoptosis. B: Panels 1–4, micrographs of primary corticohippocampal neuron cultures visualized by hematoxylin and eosin (H&E) staining: panel 1, untreated control primary cortico-hippocampal neuron cultures; panel 2, cultures treated with hrC5a (100 nM for 24 hr); panel 3, neuronal cultures 24 h after glutamate; panel 4, cultures pretreated for 24 h with hrC5a showed marked reduction of damaged neurons (small arrow, neurons with intact projection). Scale bar = 20 μm.

caspase-3 is activated by glutamate or controlled by hrC5a treatment. It has been suggested that activation of the caspase-3 proenzyme may be associated with an imbalance of Ca^{2+} metabolism and oxidative stress during glutamate receptor activation in the initial phase of excitotoxic death cascades [Choi, 1995]. We note that C5a may play an important role in Ca^{2+} mobilization in astrocytes and neuroblastoma cells (G.M. Pasinetti, unpublished observation).

Complement is activated in the AD brain, where it may potentiate neurodegeneration [Pasinetti, 1996; Shen et al., 1997]. Amyloid β peptide (Aβ) can activate complement in vitro (Rogers et al., 1992a; Velazquez et al., 1997], with potentiation of Aβ toxicity under specific conditions [Oda et al., 1995; Rogers et al., 1992b]. Thus, suppression of complement activation has been considered a therapeutic target for AD [Aisen, 1997]. However, the present results suggest that C5a may have multiple effects relevant to neuronal survival. In view of the evidence that in AD brain excitotoxicity may be responsible for a widespread pattern of neurodegeneration [Olney et al., 1997], our data suggest that anaphylatoxin C5a may play an important protective role against neurodegeneration in AD.

In conclusion, our results suggest that the role of complement-derived peptides in neurodegenerative mechanisms is complex. Neuroprotection against glutamate excitotoxicity does not necessarily mean that C5a is neuroprotective against other toxic mechanisms relevant to AD. Further elucidation of the activities of anaphylatoxin in brain is essential to the search for specific immunomodulatory/anti-inflammatory strategies to promote neuronal survival in AD.

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