RGD Domains Neuroprotect the Immature Brain by a Glial-Dependent Mechanism

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Objective: Integrin binding to extracellular matrix ligands, including those presenting RGD motifs, modulate diverse cellular processes. In the brain, many endogenous RGD-containing molecules are induced after damage. Previously, the gene therapy vector termed NLSCt, which displays an RGD motif, was shown to neuroprotect after immature brain excitotoxicity. We analyze whether neuroprotection is mediated by the RGD motif.

Methods: RGD-containing synthetic peptide GPenGRGDSPCA (GPen) was injected 2 hours after *N*-methyl-D-aspartate–mediated excitotoxicity to the postnatal day 9 rat brain. Damage and glial/inflammatory response were evaluated 3 days later. In addition, the neuroprotective effect of GPen and NLSCt after *N*-methyl-D-aspartate–induced cell death was also analyzed in vitro using neuron-purified and mixed neuron-glia primary cultures. To further characterize whether the neuroprotective effect was mediated by glial-derived soluble factors, we also tested the protective ability of conditioned media from RGD-treated microglia, astrocyte, or mixed glia cultures.

Results: Animals treated with GPen peptide showed functional improvement, a significant reduction in lesion volume up to 28%, and a decrease in the number of degenerating neurons. In addition, *N*-methyl-D-aspartate–injected animals treated with both RGD-containing molecules at the neuroprotective doses showed a significant increase in microglial reactivity and microglia/macrophage cell number, but no differences in neutrophil infiltration and the astroglial response. Finally, in vitro studies showed that the neuroprotective effect was observed in mixed neuron-glia, but not in neuron-purified cultures. Conditioned media from RGD-treated microglial, astroglial, and mixed-glial cultures were not protective.

Interpretation: These results suggest that RGD-containing molecules neuroprotect by a glial-dependent mechanism.

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Brain damage as a consequence of perinatal cerebral hypoxia/ischemia is a major cause of acute mortality and severe chronic disabilities. Several lines of evidence suggest that in comparison with the adult brain, the immature brain responds in a particular fashion to brain injuries,[1,2] partly because many adult gene expression patterns, neural circuits organization, cell differentiation, and myelination have not yet been completed.[3] In recent years, we have contributed to the characterization of the neurodegenerative process and associated glial, oxidative, and inflammatory response after excitotoxic brain damage to the postnatal brain,[4–7] and have developed several pharmacological and gene therapy strategies for neuroprotection.[8–10] We have shown that nonviral gene therapy using protein vectors constitutes a promising tool for the introduction of therapeutic genes, and particularly we have used a recombinant vector termed NLSCt for gene delivery to the damaged postnatal central nervous system (CNS).[10–12] This vector uses β-galactosidase as scaffold,[13,14] holds a polylysine tail for DNA attachment, displays an integrin-interacting motif from the GH loop region of the foot-and-mouth disease virus (FMDV) displaying a prototypical three–amino acid Arg-Gly-Asp (RGD) integrin-interacting motif,[15] and bears a short peptide sequence acting as nuclear localization signal.[16] We found that NLSCt carrying the control GFP gene was neuroprotective when injected after excitotoxicity to the immature brain.[10] As integrin interaction has been implicated in diverse cellular processes, we postulate

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that this neuroprotective effect could be mediated by the interaction of the RGD domain of this vector with cell integrins present in neurons and/or glial cells.

Integrins are a complex family of molecules that can form more than 20 different glycoprotein receptors composed of one α and one β subunit, and show overlapping ligands.[17] Moreover, they transduce both outside-in and also inside-out signals,[18] and a single integrin can recognize two different ligands and transduce different signals in response to each of them.[19] In particular, in the CNS, integrins are widely but differentially expressed in distinct neuronal populations[20–22] and have been implicated in neuronal migration, survival, differentiation, synaptogenesis, and neuritic outgrowth.[23] In addition to neurons, glial cells also express a wide variety of integrins,[20,24] and integrin-mediated adhesion to extracellular matrix and other cell types can also lead to signaling events affecting cell activation, motility, proliferation, and apoptosis.[23,25] The finding that RGD, a three–amino acid sequence, was an essential cell adhesion motif,[26] and that a family of integrins could recognize RGD motifs in different extracellular matrix proteins, gave this motif a key role in cell adhesion biology.[27] In fact, small RGD peptides have been commonly used to inhibit integrin signaling. Within the CNS, extracellular matrix proteins such as vitronectin, osteopontin, fibronectin, and L1 neurite-promoting molecule present RGD domains.[23] Among the integrins that bind the RGD motif are αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1, and αIIbβ3. These integrins are present in the cell membrane of neurons, astrocytes, microglia, oligodendrocytes, endothelium, or immune inflammatory cells.[23]

Accordingly, the aim of this study was to determine the neuroprotective potential of the integrin-interacting motif RGD by injecting the cyclic RGD peptide GPen (GPenGRGDSPCA) or NLSCt after postnatal excitotoxicity. Moreover, as integrin interactions are thought to play an important role in glial cell activation and inflammatory cell recruitment, we have examined whether these RGD-containing molecules modulate the glial response to injury and influence neutrophil recruitment. To analyze the putative modulatory role of RGD-containing peptides over integrin-related signaling pathways, we have performed in vitro studies where we evaluated phosphorylation of p42/44 mitogen-activated protein kinase (extracellular signal-regulated kinases 1 and 2 [Erk1/2]), a well-known downstream effector of integrin signaling. Moreover, to determine whether the neuroprotective effect is dependent on glial cells, we used mixed neuron-glia and purified neuron primary cultures. Finally, to analyze whether the neuroprotection was dependent of a glial-derived soluble factor, we used conditioned media from RGD-treated microglial, astroglial, and mixed glial cultures.

Materials and Methods

In Vivo Studies

EXCITOTOXIC INJURY AND RGD TREATMENT PARADIGM. Experimental work was conducted according to European directives and university ethical commission. Nine-day-old Long–Evans black-hooded rat pups (15–20gm; Janvier, Le Genest Saint Isle, France) were used. Excitotoxic lesions were performed as described previously.[5] In brief, under isoflurane (Baxter International, Deerfield, IL) anesthesia, 18.5nmol *N*-methyl-D-aspartate (NMDA) in 0.15μl saline solution (0.9% NaCl) were injected into the sensorimotor cortex using a stereotaxic frame. Two hours later, 1μl of either GPen-GRGDSPCA (GPen, 7–1,000μM; Gibco BRL, Gaithersburg, MD), NLSCt vector mixed with pEGFP plasmid (herein NLSCt, 7μM), the nude NLSCt protein vector (herein nNLSCt, 7μM), or vehicle (NaCl 0.9%) was injected at the same coordinates. In vivo studies were designed into two independent series of experiments involving several litters and mixing experimental groups in each litter (Table).

BEHAVIOR AND NEUROLOGICAL TESTING. Quantitative methods for the evaluation of adult motor performance were

Table. **In Vivo Experimental Design**

Experimental Series	Treatments	Animals Used (12-hour survival time), n	Animals Used (3-day survival time), n
1	NMDA + saline	4	18
1	NMDA + NLSCt 7μM	5	21
1	NMDA + nNLSCt 7μM	—	4
2	NMDA + saline	5	12
2	GPen 7μM	—	9
2	GPen 100μM	—	4
2	GPen 1mM	5	8
Total		19	76

NMDA = *N*-methyl-D-aspartate; GPen = GPenGRGDSPCA.

adapted for postnatal pups (postnatal days 9–12 [P9–P12]) as described previously[10] (see supplementary material for details).

HISTOLOGY AND LESION VOLUME MEASUREMENT. Twelve hours or 3 days after lesion, rats were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were postfixed, cryoprotected, and frozen with dry CO_2. Parallel cryostat coronal sections (30μm thick) of the entire brain, separated by 240μm (every eighth section), were stained for Nissl and used for the measurement of hemisphere volume and lesion volume as described previously.[10] Neuronal degeneration in the secondary degenerating hippocampus was detected by Fluoro-Jade B staining.[10]

IMMUNOHISTOCHEMISTRY AND HISTOCHEMISTRY. Sections were processed for astroglial and neutrophil labeling by using anti–glial fibrillary acidic protein (GFAP; 1:1,800; Dakopatts-Z0334; Dakopatts, Glostrup, Denmark) or anti-myeloperoxidase (MPO) (1:400; Dakopatts-A0398), respectively. Anti–rabbit horseradish peroxidase–labeled antibody (1:200; NA934V; Amersham Biosciences, Buckinghamshire, United Kingdom) was used. Microglia/macrophages were demonstrated using biotinylated Lycopersicon esculentum (tomato) lectin[28] (6μg/ml; Sigma-L9389; Sigma, St. Louis, MO) followed by avidin-peroxidase (Dakopatts, PO364). Diaminobenzidine developing procedure was used in both cases.

DENSITOMETRY AND CELL NUMBER QUANTIFICATION. For densitometrical measurements, analySIS software was used. Three 20× images from ipsilateral and contralateral cortices of three different sections corresponding to −0.3, 0 (injection level), and +0.3mm coordinates from bregma conformed the region of interest (ROI). The cortical area surrounding the injection level was defined as the lesion core. As described previously,[10] mean gray value labeling density of ROI (GVM) and the area occupied by immunoreactive cells within ROI were obtained for each section. To analyze the immunoreactive area within ROI, we set the threshold qualitatively according to the immunohistochemical signal in one image, and it was maintained unchanged for the rest of the images. Tomato lectin (TL)+ microglia/macrophage and MPO-positive cell numbers were counted in 20× micrographs from sections corresponding to the previously detailed coordinates. Fluoro-Jade B–positive cells were counted in 20× micrographs from two different areas of the ipsilateral hippocampal CA1 region.

In Vitro Studies

CELL CULTURES. Primary cortical cultures were prepared from E17 OFA rat embryos by dissecting cerebral cortices free of meninges and plating cells at a density of 1.8×10^6 cells/ml in Basal Medium Eagle (BME) (04-25050; Pan Biotech, Aidenbach, Germany) supplemented with 5% fetal horse serum (Invitrogen, La Jolla, CA), 5% fetal calf serum (Invitrogen), 2mM glutamine, 0.6% glucose, 50U/ml penicillin, and 50μg/ml streptomycin on 10μg/ml polylysine-coated wells. At 7 days in vitro (DIV), mixed neuron-glia cortical cultures were incubated with the same mixture but using 10% fetal horse serum instead of 5% fetal calf serum + 5% fetal horse serum and adding 10μM cytosine arabinoside. Under these conditions, at 10 to 12 DIV when cultures were used, they contained 21 ± 2% astrocytes (GFAP-positive cells) and 11 ± 2% microglial cells (NDPase-positive cells). Purified cortical neuron cultures were prepared in the same manner, but after dissociation, cells were plated at the same density in Neurobasal medium (Invitrogen) supplemented with 2% B27 supplement (Invitrogen), 0.5mM GlutaMaxI (Invitrogen), 100U/ml penicillin, and 100μg/ml streptomycin. In this case, 3μM cytosine arabinoside was added at 4 DIV. Under these conditions, at 10 to 12 DIV when cultures were used, they approximately contained 97% of neuronal cells and less than 3% glia. Mixed glial cultures were prepared from Bl/C57 mouse neonates. Meninges-free cerebral cortices were dissected out and dissociated by trypsinization and repeated pipetting. Cells were plated at 3×10^5 cells/ml in Dulbecco's minimum essential medium:F12 medium (31330-038; Invitrogen) supplemented with 10% fetal calf serum and antibiotics as described earlier. Cultures were used at 15 to 18 DIV and consisted of approximately 75% astrocytes (GFAP-positive cells) and 25% microglia (Tomato lectin–positive and $CD11b^+$). Microglial purified cultures, prepared from mixed glial cultures by mild trypsinization as described previously,[29] were used 24 hours after microglia isolation and consisted of 97.4 ± 0.9% microglia and <1% astrocytes.

Mixed neuron-glia cortical cultures and purified cortical neuron cultures were treated with 300μM NMDA in the culture medium for 20 and 10 minutes, respectively. After washes, cells were incubated with different concentrations of GPen (0.1–1,000μM), nNLSCt (0.001–1μM), or K10lacZ (0.001–1μM) in 200μl OPTIMEM medium (Invitrogen). The K10lacZ molecule was used as a negative control because it consists of the nNLSCt molecule without the RGD motif.[14] After 1 hour, 800μl of the non-RGD control medium described earlier for each culture type was added to each well, and the cultures were further maintained for 72 hours before fixation with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). For conditioned media experiments, astrocyte-purified cultures,[29] microglia-purified cultures, and mixed glial cultures[30] were treated with GPen or nNLSCt and incubated for 24 hours in supplemented Neurobasal medium as described earlier.

NEURONAL CELL VIABILITY. Purified neuron cortical cultures were evaluated for neuronal viability using the spectrophotometric 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) assay by incubating cultures for 1 hour at 37°C with 5% CO_2 and 0.2mg/ml MTT. Cells were then lysed in dimethylsulfoxide and MTT product (colored formazan) was determined by measuring absorbance at 570 and 630nm, and the 570-630 subtracted data were used. In mixed neuron-glia cortical cultures, evaluation of neuronal cell viability was achieved by immunocytochemical labeling with microtubule-associated protein 2 (MAP2; 1:1,500; MAB3418; mouse monoclonal; Chemicon, Temecula, CA), anti–mouse HRP-linked (1:200; NA931V; Amersham Bioscience) as secondary antibody and diaminobenzidine tetrahydrochloride-hydrogen peroxide developing procedure.

MAP2-positive cells were counted in eight 40× fields in each well.

DATA PROCESSING AND STATISTICAL ANALYSIS. All results are expressed as mean ± standard error of the mean. Analysis of variance followed by Fisher's partial least squares difference post hoc test was used to determine significant differences ($p < 0.05$) in lesion volume, cell counts, and densitometry. Repeated-measures analysis of variance followed by Fisher's partial least squares difference post hoc test was used to evaluate significant differences ($p < 0.05$) between groups in inclinated grid walking.

Results

Neuroprotective Effect of RGD-Containing Molecules after Excitotoxicity to the Immature Brain

NMDA injection into the sensorimotor cortex of postnatal day 9 rats induced neurodegeneration both in the primary lesioned cortex and in secondary lesioned areas such as the caudal subplate neuronal layer, striatum, and hippocampus.[5] When rats were injected, 2 hours after lesion, at the same location with 7μm NLSCt vector linked to a control plasmid (NLSCt), a pronounced neuroprotection was observed 3 days after (28 ± 6% protection) (Fig 1A). Interestingly, when nNLSCt (the nude vector) was injected under the same circumstances, it also induced significant neuroprotection (28 ± 5% protection) (see Fig 1A). Moreover, when the cyclic RGD-containing peptide GPen was injected at different doses 2 hours after lesion, a dose-dependent neuroprotective effect was clearly observed (25 ± 8% reduction in the percentage of lesioned hemispheric volume at 1mM) (see Fig 1B). To evaluate neurodegeneration in secondary distal areas, we counted Fluoro-Jade B–positive degenerating neurons in the hippocampal CA1 (Fig 2). Quantification showed a significant decrease in the number of Fluoro-Jade B–positive neurons at two different levels of the CA1 hippocampal layer in animals treated with GPen, NLSCt, or nNLSCt.

As shown by functional evaluation of the lesioned animals performed by the inclinated grid test, NMDA lesioned animals showed a significant decrease in the total time spent in the grid in comparison with nonlesioned saline-injected animals (see Fig 1C). Animals treated with GPen (1mM) showed an important progressive functional recovery after the lesion, being statistically indistinguishable from the nonlesioned animals at 3 days after lesion (see Fig 1C). This functional improvement, however, was not observed in animals treated with NLSCt (7μM).

Neuroprotection Mediated by RGD-Containing Molecules Is Associated with a Modulation of the Glial Response

When compared with control NMDA + saline–injected animals, NMDA-injected animals treated with the neuroprotective doses of either GPen (1mM) or NLSCt (7μm) showed significant differences in the microglial/macrophage response at 12 hours after lesion (Figs 3A–C). Microglia/macrophage cell number was greater in

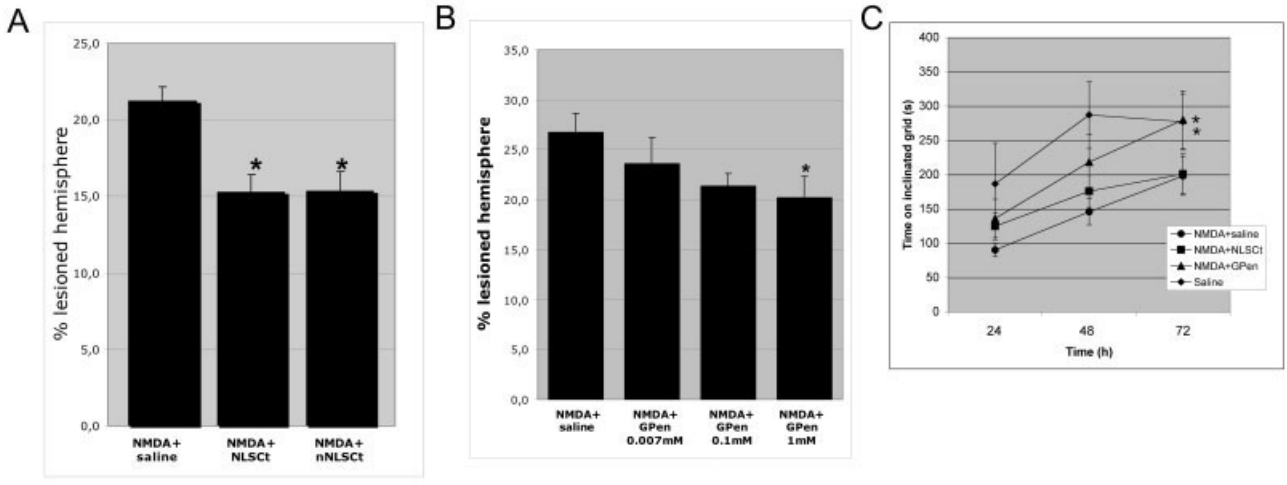

*Fig 1. RGD-containing molecules are neuroprotective after in vivo excitotoxic injury. Administration of NLSCt or the nude vector (nNLSCt) 2 hours after lesion showed, after 3 days, a similar significant (*p < 0.05) reduction in the percentage of lesioned hemisphere when compared with* N*-methyl-*D*-aspartate (NMDA) + saline–injected animals (A). Interestingly, in a separate series of experiments, when lesioned animals were injected with the RGD peptide GPen under the same conditions, a dose-dependent decrease in the percentage of lesioned hemisphere was observed when compared with NMDA + saline–injected animals (*p < 0.05). Functional evaluation of these animals using the inclinated grid test showed that lesioned animals treated with GPen (1mM) recovered significantly better from the lesion in comparison with NMDA + saline–injected animals, reaching the level of the nonlesioned saline-injected animals after 3 days (C, *p < 0.05).*

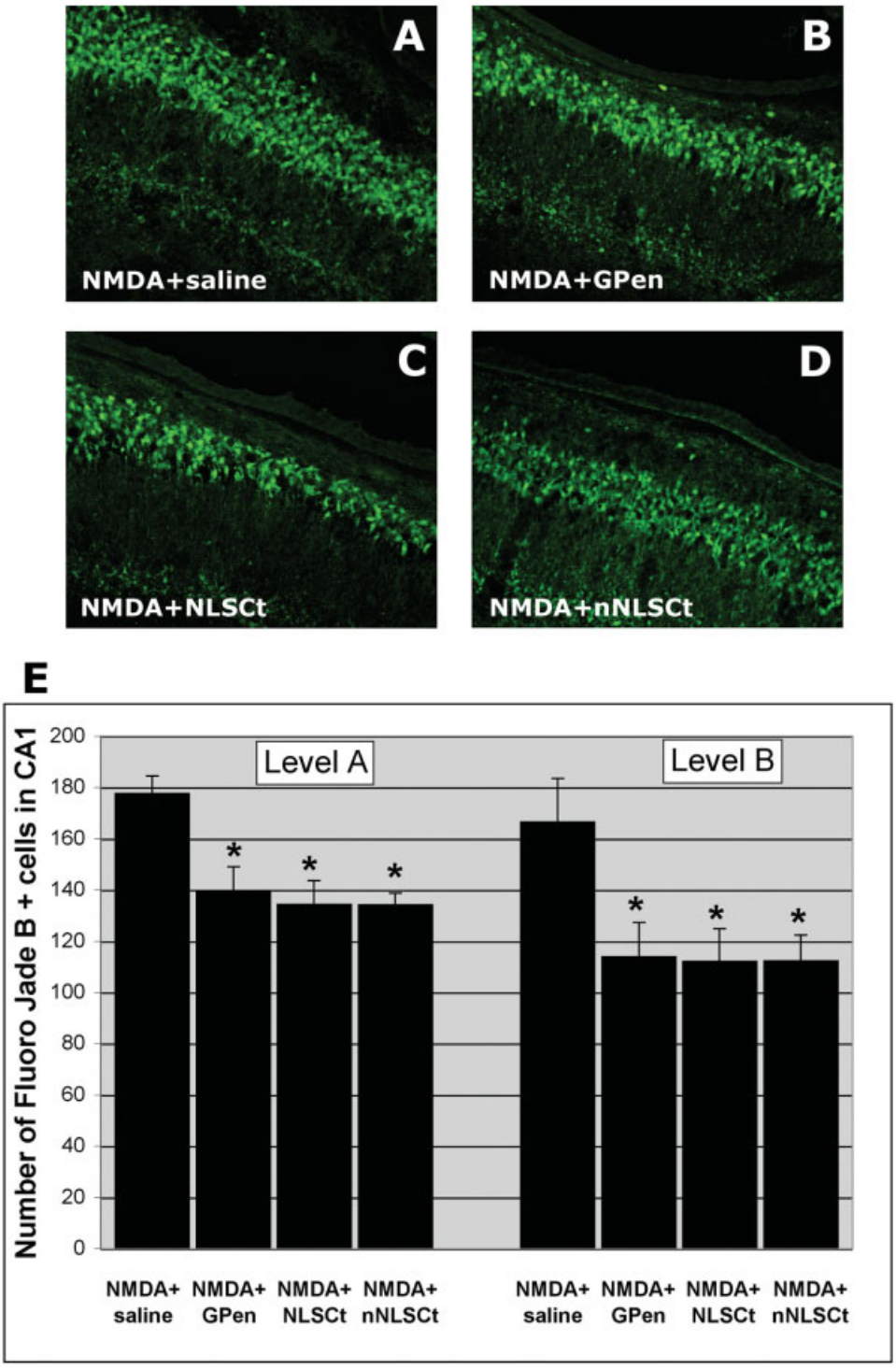

Fig 2. RGD-containing molecules induce neuroprotection at CA1. Two hours after lesion administration of the RGD peptide GPen (1mM) (B), NLSCt (7μM) (C) or nNLSCt (D) showed, after 3 days, a significant reduction ($p < 0.05$, when compared with* N-*methyl-D-aspartate [NMDA] + saline–injected animals; A) in the number of degenerating neurons stained with Fluoro-Jade B in the CA1 layer of the hippocampus. This reduction was observed at two different zones of the caudal CA1 layer at two rostrocaudal levels from the lesion core (level A: bregma −2.43; level B: bregma −2.70) (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]*

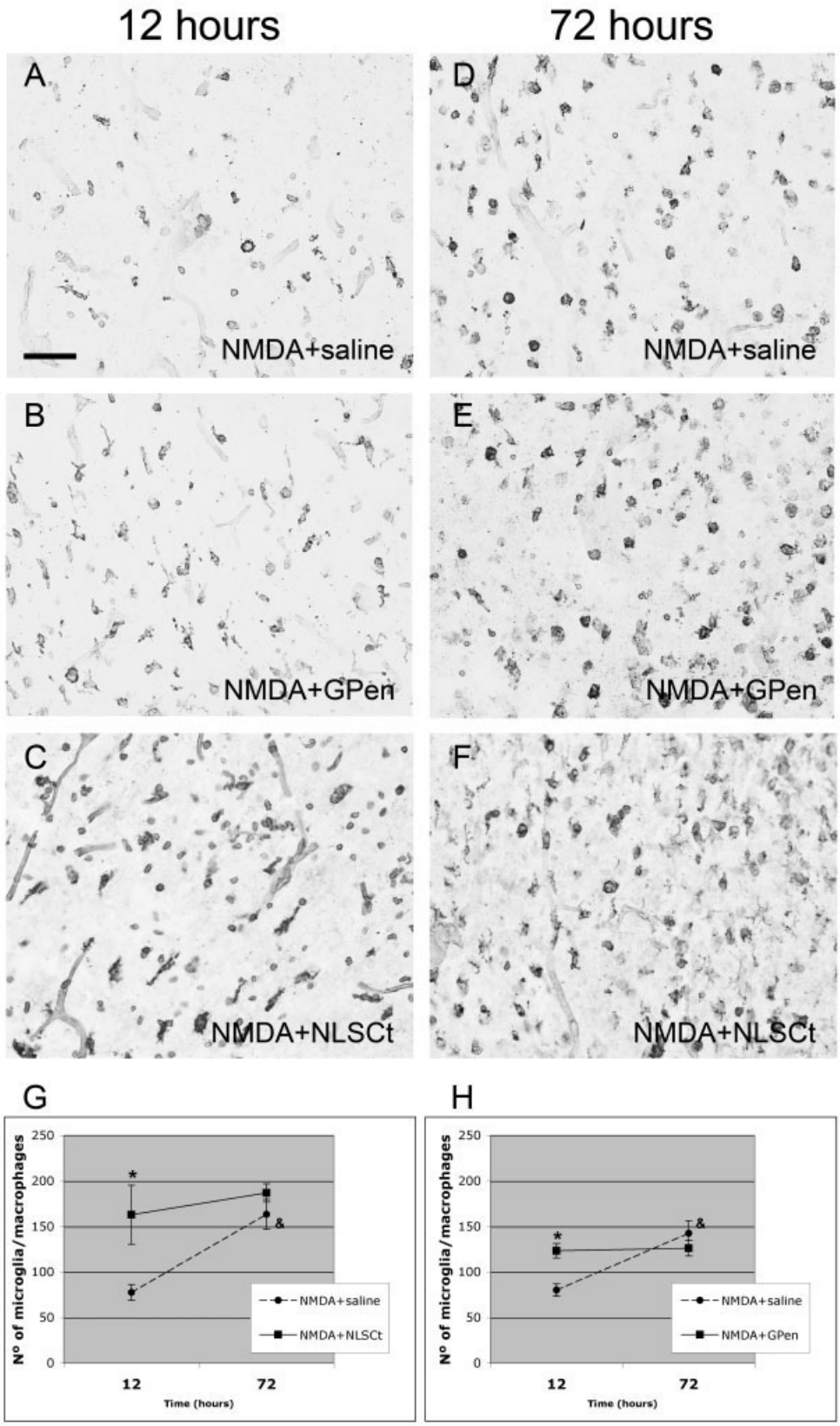

Fig 3. RGD-containing molecules modify microglial/macrophage reactivity after excitotoxic injury. Administration of GPen (1mM, B) or NLSCt (7μM, C) showed a significant variation in microglial/macrophage reactivity observed in the lesion core (cortical area surrounding the injection level) at 12 hours in comparison with N*-methyl-*D*-aspartate (NMDA) + saline animals (A), which was not evident at 72 hours (D–F). Quantification of the number of tomato lectin microglia/macrophages (G, H) showed an increase at 12 hours after lesion in both treatments (*$^{*}p < 0.05$ *and* $^{\&}p < 0.05$ *compared with NMDA + saline at 12 hours). Scale bar = 50μm.*

the lesion core of NLSCt- (see Fig 3G) and GPen-treated (see Fig 3H) animals. Accordingly, tomato lectin densitometry and the area of ROI occupied by reactive microglia/macrophages were also increased with GPen or NLSCt treatment (not shown). At 3 days after lesion, when characteristic NMDA-induced increase in microglial/macrophage reactivity is observed (see Figs 3D, G, H), the number of microglia/macrophages found was similar in GPen, NLSCt, and control NMDA + saline–injected animals (see Figs 3E–H). In contrast, no changes in the number of infiltrated neutrophils or remarkable morphological or densitometrical differences regarding GFAP-positive astroglial reactivity were observed (see Supplementary Fig 1).

Neuroprotective Effect of RGD-Containing Molecules after Excitotoxicity In Vitro

Mixed neuron-glia cortical cultures containing 21 ± 2% astrocytes and 11 ± 2% microglial cells were treated with either GPen or nNLSCt both under basal conditions and after NMDA-induced neurodegeneration. Addition of nNLSCt to mixed neuron-glia cultures under basal conditions did not affect neuronal survival (Fig 4A) as observed by direct MAP2-positive cell counts. However, at 72 hours after NMDA treatment, the low concentration of 0.01μM nNLSCt reverted excitotoxic neuronal death but did not show any effect at greater concentrations (see Fig 4B). The addition of K10lacZ (control vector without RGD motif) did not show significant neuroprotection (see Fig 4B). Like nNLSCt, the GPen peptide did not affect neuronal survival under basal conditions (see Fig 4A) but did show complete protection from excitotoxicity when added after NMDA treatment (see Fig 4B). As observed in vivo, the degree of neuroprotection observed for GPen and NLSCt differs, being necessary 10,000 times more concentrated than GPen to provide maximum neuroprotection. Neuron-glia cortical cultures showed a rapid and transient phosphorylation of Erk1/2 after exposure to GPen or nNLSCt, indicating activation of intracellular signaling cascades (see Supplementary Fig 2).

To confirm whether the neuroprotective effect was mediated directly by the RGD domains on neuronal cells, we repeated the experiments on purified cortical neuron cultures containing less than 3% of glia. Neither GPen nor nNLSCt induced significant effects when added for 3 days on neuron-purified cultures under basal conditions (data not shown). Moreover, both GPen and nNLSCt failed to show neuroprotection when neuron-purified cultures were previously treated with NMDA (see Fig 4C).

To analyze whether glia-dependent neuroprotection was mediated by diffusible factors released by glial cells into the culture medium, we performed conditioned media experiments by treating purified astrocyte cultures, purified microglial cultures, or mixed glial cultures with the neuroprotective doses of GPen or nNLSCt. Conditioned media were then applied to purified neuronal cultures previously treated with NMDA (Fig 5). There was a significant increase in neuronal survival when cultures were incubated with control astrocyte, microglia, and mixed glia conditioned media. However, no differences were observed between the control conditioned media and the conditioned media from astrocyte, microglia, or mixed glial cultures treated with GPen or nNLSCt (see Fig 5).

Discussion

This work demonstrates that RGD domains (including small cyclic peptides such as GPen) are neuroprotective against excitotoxic damage in vivo and in vitro, point-

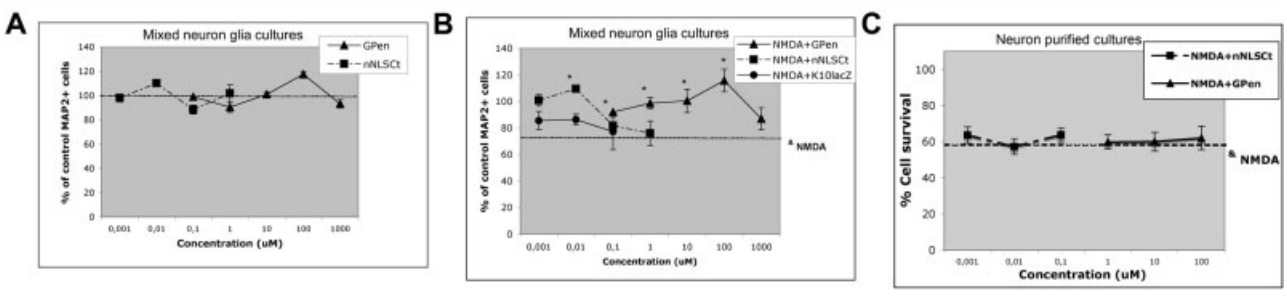

Fig 4. RGD-containing molecules are neuroprotective after in vitro excitotoxic injury. Mixed neuron-glia cortical cultures were exposed to saline (A) or N*-methyl-*D*-aspartate (NMDA) (B), and then incubated at the indicated concentrations of NLSCt, GPen, or saline. NMDA caused a significant decrease in neuronal viability after 72 hours (*$^{\&}p < 0.05$, dotted line *in B and C). Although no effect was observed with NLSCt or GPen under basal conditions (A), a significant decrease in neuronal cell death was observed by microtubule-associated protein 2 (MAP2)–positive cell counts in NMDA-treated cultures incubated with NLSCt or GPen (B). Moreover, cultures treated with the negative control K10lacZ did not show any neuroprotection (B). When purified cortical neuron cultures were exposed to saline, or to the most effective concentrations of NLSCt or GPen after NMDA treatment, no significant increase in neuron viability was observed by 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) measurement (C).*

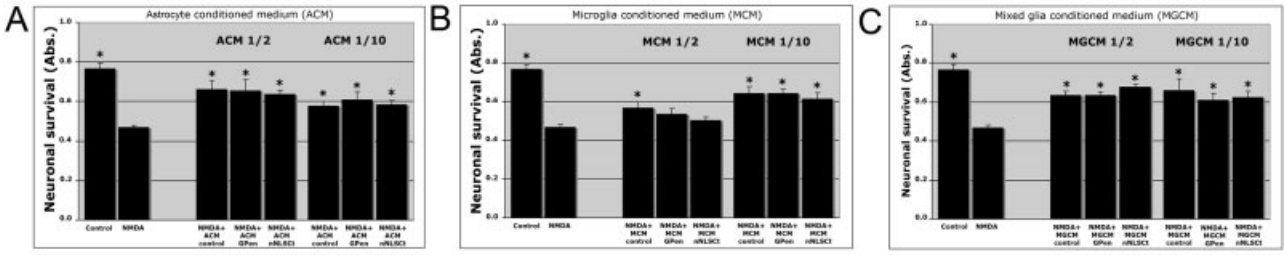

Fig 5. Conditioned media from RGD-treated glial cells is not neuroprotective after in vitro excitotoxic injury. Conditioned media from GPen- or nNLSCt-treated purified astrocyte cultures (ACM) (A), purified microglial cultures (MCM) (B), or mixed glial cultures (MGCM) (C) were tested at two different dilutions (1/2 and 1/10) for neuroprotective activity in N-*methyl-*D*-aspartate (NMDA) pretreated purified cortical neuronal cultures. Three days after addition of conditioned media, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) measurements showed that control conditioned media from astrocytes, microglia, and mixed glial cultures protect against NMDA-induced neuronal cell death, but neither GPen nor nNLSCt increases this neurotrophic activity (*$p < 0.05$ compared with NMDA).*

ing toward an important neuroprotective role of endogenous RGD-containing molecules after acute lesions to the CNS. Interestingly, this neuroprotective effect of RGD domains was associated with important effects on glial cells, including a rapid activation of microglial reactivity in vivo and the fact of glial dependence for neuroprotection in vitro. Moreover, RGD domains may exert their effects not only by inhibiting the interaction of integrins with their natural ligands as previously thought, but also by directly activating outside-in integrin signaling cascades. Finally, we show here that RGD domains are neuroprotective in an in vivo postnatal brain injury model, and preliminary results (O. Campuzano, personal communication) suggest that the GPen and NLSCt molecules are also neuroprotective in adult rats, pointing to a general neuroprotective role of RGDs.

The GPen peptide, a cyclic form of RGDS[31] that shows a higher binding specificity and a lower inhibitory concentration of 50% than linear RGD, is an efficient inhibitor of αVβ3- and αVβ5-mediated cell attachment to vitronectin but shows nearly no activity toward attachment on fibronectin by α5β1.[31] Accordingly, GPen may, at least in part, exert its neuroprotective effect by interacting with αVβ3 and αVβ5, either inhibiting their binding to the extracellular matrix or activating intracellular mechanisms. In particular, in monocytes and macrophages, αvβ3 integrin expression is involved in movement, transendothelial migration, chemotaxis, and production of free radicals after induction of the respiratory burst,[25] and it contributes to the macrophage/microglial phagocytosis of affected neurons, a process that can be partly inhibited by RGDS peptides.[32] Moreover, αVβ3 is up-regulated in astroglial cells after adult focal stroke, whereas its main ligand, osteopontin, is expressed by surrounding microglial cells at early phases of the degenerative process,[33] suggesting that αVβ3-osteopontin binding may play a role in glial cell activation, migration, and tissue remodeling after brain ischemia. Furthermore, a recent study has shown that osteopontin treatment is neuroprotective after oxygen/glucose deprivation in cortical neuron cultures and when administered intracerebroventricularly before and after transient middle cerebral artery occlusion.[34] In fact, neuroprotection by osteopontin in these cortical neuronal cultures was dependent on the osteopontin RGD motif and mediated by activation of phosphatidyl inositol-3 kinase/Akt (PI3K/Akt) and Erk1/2 signaling pathways,[34] which together with focal adhesion kinase and nuclear factor-κB mediate integrin outside-in intracellular signaling.[25] In agreement, we show that both NLSCt and GPen activated Erk1/2 in mixed neuron-glia cultures, only at the neuroprotective doses (see supplementary material), although in our experimental paradigm we cannot conclude whether this pathway is mediating the neuroprotective effect observed.

In addition, GPen also inhibits the adhesion of denatured collagen or fibrinogen to immobilized platelet αIIbβ3 integrin, implying that GPen could also interact with αIIbβ3 integrin.[35] In fact, αIIbβ3 acts as a functional receptor for fibrinogen, but also for vitronectin, fibronectin, and vascular adhesion molecules. It also supports platelet aggregation and thrombus formation, being therefore a target for antithrombotic therapy, as indicated in a study where intravenous injection of an αIIbβ3 antagonist showed beneficial effects after ischemic damage.[36]

Significantly, although both RGD-containing molecules were neuroprotective, the effects of NLSCt protein and GPen peptide administration were not identical. The different neuroprotective profiles of GPen and NLSCt could be explained by several reasons. First, it should be noted that the effective concentration of the two molecules was different, being necessary to use a thousand times greater concentration of GPen than that of NLSCt to exert neuroprotection after an excitotoxic injury. In agreement, it has been demon-

strated that cyclic RGD peptides in the context of larger proteins show a 1,000-fold increase in their αvβ3 integrin binding affinity.[37] Accordingly, linear peptides inhibit FMDV infection in the millimolar range, whereas longer peptides, like several GH loop peptides as that in NLSCt, do the same but at an inhibitory concentration of 50% of 0.8μM (see Mateu and colleagues[38] for review), a similar concentration at which we observed the neuroprotective effects of NLSCt. Second, conformational effects could also explain the slight differences observed between these two molecules, as the RGD domain in the GH loop of NLSCt holds a higher degree of flexibility. Third, the slightly different integrin specificity between NLSCt and GPen could also account for those differences. The RGD motif in the NLSCt protein mediates FMDV attachment to the cell membrane leading to viral infection[39] by interacting with integrins αvβ3,[40] αvβ6,[41] αvβ1,[42] and αvβ8,[43] which have been shown to act as receptors for the FMDV, and the infection of cells expressing these integrins can be inhibited by RGD peptides or anti-αv antibodies. In addition, although it is controversial whether α5β1 integrin acts as a receptor for FMDV,[40,42] the GH loop of FMDV inserted into another macromolecule including β-galactosidase enables this protein to bind to this integrin,[15] and thus we cannot discard that NLSCt also binds to α5β1. Therefore, we cannot rule out that, in addition to αVβ3, other integrins could also contribute to the neuroprotective effect observed. In fact, the GPen interaction with other αV integrins besides αVβ3 has not been completely established, and thus considering that NLSCt also recognizes αVβ1, αVβ6, and αVβ8, it is possible that these integrins are also involved and account for the increased neuroprotection observed with NLSCt in comparison with GPen. And finally, when trying to comprehend the consequence of integrin binding to its ligands, it must be noted that the resulting effect is highly dependent on the ligand itself. For example, although αvβ3 binds to vitronectin and fibronectin, engagement of αvβ3 to vitronectin (but not to fibronectin) induces PI3K/Akt signaling activation and association of PI3K to β3 integrin.[19] Moreover, inside-out signaling can also alter ligand specificity of a given integrin. For instance, cells that under basal conditions attach to vitronectin and fibronectin via αvβ3 will only attach to vitronectin when activated.[19]

Another relevant point of discussion is whether RGD motifs are interacting with neurons, glia, and/or inflammatory cells. On one hand, RGD peptides could be acting directly on neuronal cells, as vitronectin has been shown to induce neuronal differentiation,[44] and RGD peptides can induce upregulation of neurotrophins such as brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor and expression of their receptors TrkB and TrkC in hippocampal slices.[45] In this sense, interaction of β1 integrins with laminin in neuronal cultures activates the prosurvival pathway PI3K/Akt, and blockade of these interactions renders neurons more susceptible to excitotoxicity.[46] On the other hand, it should be noted that whereas no neuroprotection was observed after either NLSCt or GPen treatment in neuron-purified cortical cultures with minor glial cell presence, a neuroprotective effect was achieved with both RGD-containing molecules after excitotoxicity in cortical mixed neuron-glia cultures with around 32% of glial cells. This finding leads to the hypothesis that RGD domains mediate integrin signaling in glial cells, triggering the release of neurotrophic or other factors, which in combination with integrins modulate growth factor receptor signaling,[17] as occurs in other neuronal culture systems.[47] However, conditioned media from GPen- or nNLSCt-treated astrocytes, microglia, or mixed glial cultures did not protect purified neuronal cultures against excitotoxicity, suggesting that the glial neuroprotective effect may depend on cell contact. Interestingly, the most striking effect of both RGD-containing molecules in glial cells was the increase in microglial/macrophage reactivity and cell numbers after the excitotoxic lesion, a phenomenon that has classically been linked to exacerbation of damage, but its role has been revisited in recent years. Although the effects of αv blocking on endogenous microglial cells are unknown, αvβ3 integrins are important to monocytic transendothelial migration under inflammatory conditions, and either αv antibodies or deficiency in β3 subunit have been shown to reduce monocyte transendothelial migration,[48] further implying endogenous microglial cells in the neuroprotective effect. Regarding the infiltration of inflammatory cells, especially neutrophils, which are associated with tissue damage exacerbation, it has been described that the interaction of this cell type with extracellular matrix can be potently inhibited by antibodies against β3 in epithelial cell cultures.[49] However, in our in vivo excitotoxic damage model, RGD motifs do not reduce the entrance of neutrophils after the lesion; thus, this mechanism cannot account for the neuroprotection observed.

In conclusion, this study shows a glial-dependent neuroprotective action of RGD domains, which is accompanied by an increased reactivity of microglial cells, suggesting that the activation and/or inhibition of particular integrins could modulate the glial and inflammatory response after nervous system lesions. The exact mechanisms underlying this neuroprotection and the possible participation of other cell types need further research. Finally, the results obtained in this study give support to the use of small cyclic RGD peptides as neuroprotectants.

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