Evidence for Neuroprotective Effects of Acidic Fibroblast Growth Factor in Alzheimer Disease

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Abstract. Recent studies indicate that fibroblast growth factors (FGFs) might confer neuroprotection against excitotoxicity. Therefore, the fact that acidic FGF (aFGF) is more abundant in motoneurons than in the hippocampal formation suggests that aFGF contributes to the selective vulnerability of neurons in entorhinal cortex (EC) in Alzheimer disease (AD). In order to understand the role of aFGF in AD, patterns of aFGF, FGF receptor (FGFR), and N-methyl-D-aspartate (NMDA) receptor (NMDAR) expression in the EC and hippocampus of AD and control cases were investigated, and effects of aFGF on excitotoxicity were examined in vitro. In AD, the number of aFGF immunolabeled neurons was decreased in EC, while the remaining neurons showed significantly higher aFGF immunoreactivity. This latter group of neurons did not show cytoskeletal abnormalities. Acidic FGF and FGFR immunoreactivity were positively correlated, whereas a negative correlation was found between aFGF and NMDAR expression. These results were confirmed in vitro utilizing NT2N cells. Higher levels of FGFR protein were expressed in aFGF-treated cells, while less NMDAR protein was found compared with untreated cells. Furthermore, exposure of treated and untreated NT2N cell to glutamate revealed that aFGF can prevent glutamate induced cell death.

Taken together these data suggest that aFGF regulates the expression of NMDAR and FGFR and thereby contributes to neuroprotection against glutamate excitotoxicity. Therefore, altered patterns of aFGF immunoreactivity in EC in AD are an important marker for selective vulnerability of EC neurons.

Key Words: aFGF; Alzheimer disease; Excitotoxicity; Neuroprotection, NMDA receptor; NT2N cells.

INTRODUCTION

Alzheimer disease (AD) is a neurodegenerative disorder that results in neuronal/synaptic loss, neurofibrillary tangles, and amyloid deposits (1–3). The underlying mechanisms responsible for the neurodegenerative process are not fully understood, but it is believed that increased toxicity and/or a loss of neuroprotective factors render neurons susceptible to neurodegeneration. For example, a decrease in glutamate transporter activity has been found to be associated with synaptic/neuronal damage in AD (4, 5), while overstimulation of N-methyl-D-aspartate (NMDA) receptor (NMDAR) leads to neurodegeneration supporting the possibility that excitotoxicity plays an important role in the pathogenesis of AD (6–9). In this context, it is of great interest that neurons in the entorhinal cortex layer 2 (EC2) are most vulnerable and first affected in AD, while the degenerative process does not affect other neurons (e.g. motoneurons). Even neurons in the CA3 region of the hippocampus (HC) are relatively resistant (1). This suggests that regionally produced factors might confer vulnerability to, or neuroprotection against, potentially toxic factors that promote neurodegeneration. In this regard, recent studies have shown that growth factors such as fibroblast growth factor (FGF) protect against neurodegeneration induced by axotomy (10) or neurotoxins such as excessive glutamate (11–13) and β-amyloid (14). Interestingly, acidic FGF (aFGF, also referred to as FGF 1 (15, 16) is highly expressed in motoneurons in mouse and rat brain, although very little or no aFGF is found in the hippocampal formation (i.e. EC, HC) (17–19) indicating that constitutively low expression of FGF might render EC 2 neurons susceptible to excitotoxicity. The mechanisms by which FGF protects against neurotoxicity are not completely understood. Some studies have shown that FGF modulates Ca++ influx (11, 14, 20) and might regulate expression of FGFR (FGFR) (21–23) and NMDAR protein (20, 24).

aFGF is a member of the FGF family that lacks the signal sequence important for secretion and, in contrast to the very similar basic FGF (bFGF), is predominantly expressed in the cytosol of neurons (15, 17, 25, 26). Therefore, it is important to determine whether altered expression of aFGF and/or its receptor in EC2 neurons in AD might contribute to their selective vulnerability, as well as to analyse the effects of aFGF on neuroprotective mechanisms against glutamate excitotoxicity, such as downregulation of NMDAR expression. For this purpose, neuronal expressions of aFGF, its most specific receptor FGFR3 (27), and the NMDAR subunit 1 (NMDAR1), which appears most important for glutamate mediated effects (28), were investigated in the EC and HC of AD and control cases. In addition, effects of aFGF on NMDAR1 and FGFR3 expression and cell survival following glutamate exposure were examined in a fully terminally differentiated human teratocarcinoma Ntera 2 cell line (NT2N) expressing NMDAR (29).
MATERIALS AND METHODS

Samples

A total of 15 AD cases (age 78.2 ± 1.8; 8 female, 7 male) and 10 control cases (age 69.9 ± 4.5; 5 female, 5 male) from the Alzheimer Disease Research Center at the University California, San Diego were studied within 12 hours (h) postmortem (AD: 8.5 ± 0.9; control: 4.5 ± 0.78). All AD cases had a clinical history of dementia (Blessed score: AD: 21 ± 2.4; control: 0.2 ± 0.2) and underwent detailed neurological and neuropathological evaluation. CERAD criteria were used to confirm the diagnosis of AD (30). For each case, a block from the hippocampal formation/EC was taken and fixed in 2% buffered paraformaldehyde for 72 h at 4°C. The tissue was sectioned at 40 μm with a Leica vibratome. In addition, blocks of various cortical and subcortical regions were embedded in paraffin. Cortical and subcortical blocks were sectioned and stained for routine histopathological examination. The clinical diagnosis was confirmed by the presence of plaques and neurofibrillary tangles and cases were histopathologically staged according to Braak and Braak (1).

Cell Culture

NT2 cells (Stratagene, La Jolla, CA) were maintained in DME-HG supplemented with 10% FBS, 1% L-Glutamine, and 0.1% Gentamicin. Cells (2.3 × 10^6) were then seeded in 75 cm^2 flasks and treated with 1% retinoic acid twice a week for 5 weeks (29, 31). Differentiated cells were then replated (1:6) and maintained in the above described medium supplemented with 0.1% 1-β-D-arabinofuranosylcytosine, 1% fluorodeoxyuridine, and 1% uridine (DME-HG α). After 5 days of replate I, neuronal cells were separated from non-neuronal cells by incubation in trypsin (ATV) and mechanically dislodging. Neuronal (NT2N) cells were then replated in 162 cm^2 flasks (for Western Blot analysis) and on 24 well plates or chamberslides that had been coated with Matrigel (Collaborative Biomedical, Bedford, MA) (32) and maintained in DME-HGα. After 2.5 weeks, cells were treated with aFGF (Accurate Chemicals, Westbury, NY) in different concentrations (0, 10, 25, 50 ng aFGF/ml) with and without 2.5 U/ml heparin (aFGF ± H). Heparin is known to optimize the effects of FGFs (25). After 24 h NT2N cells were either collected for Western Blot or washed twice in a controlled salt solution (120 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 15 mM glucose, 25 mM Hepes, and 50 mM glutacline; pH 7.6) and then exposed to 1 M glutamate dissolved in serum free Opti-MEM I (33) for 30 minutes (min). Control cases were exposed to Opti-MEM I and 1 row of wells per experiment was treated with a lysis solution (Promega, Madison, WI) to assess maximal cell death. In addition, control experiments were performed by selectively blocking NMDAR (MK-801, 100 nM; Calbiochem, San Diego, CA) (33), as well as adsorbing aFGF (anti-aFGF; Sigma Chemical Company, St. Louis, MO). For each treatment a row of 6 wells was used and each experiment was repeated at least 3 times.

Immunohistochemistry

Briefly, as described previously (2), vibratome sections of the HC and EC of control and AD cases were single-immunolabeled for semiquantitative evaluation with anti aFGF (monoclonal IgG; Sigma, 1:400) (34), anti FGFR3 (polyclonal IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA, 1:200) (35), anti NMDAR1 (polycional IgG, Chemicon, Temecula, CA, 1:500) (36), anti amyloid precursor protein (APP). Monoclonal IgG against human APP residues 444–592, courtesy of Athena Neurosciences Incorporated, San Francisco, CA, 1:5000) (37), and Alz 50 (monoclonal IgM against A68, courtesy of P. Davies, AECOM, 1:250). In addition, selected AD and control cases were double-labeled for aFGF (1:200)/NMDAR1 (1:200), aFGF (1:200)/FGFR3 (1:200), and aFGF (1:200)/anti tau (polyclonal IgG, Sigma, 1:75) (38). Furthermore, NT2N cells were immunolabeled with anti FGFR3 (1:200).

Optical and consistent results were obtained in vibratome sections washed for 20 min with 0.025% Triton X-100, followed by blocking with 10% normal horse serum for monoclonal and 10% normal goat serum for polyclonal antibodies. Cells were washed twice in PBS and sections, as well as cells, were incubated overnight at 4°C with the antibody/antibodies diluted as previously mentioned. For the double-immunolabeling study, control experiments were carried out to verify the specificity of the reaction by omitting one of the primary antibodies. Samples were then incubated in 1% biotinylated horse anti mouse IgG or goat anti rabbit IgG, followed by coupling (ABC Elite, Vector Laboratories) for 1 h and reacted with 0.05% Diaminobenzidine tetrahydrochloride (DAB). In the case of double-immunolabeling, sections were incubated in a mixture of FITC-conjugated goat anti rabbit IgG (1:75) and Texas red-conjugated horse anti mouse IgG (1:100) (Vector Laboratories, Burlington, CA) for 1 h. Sections were then transferred to Colorfrost slides (double-labeled sections to SuperFrost slides) (Fisher Scientific, Tustin, CA) and mounted under glass cover slips using anti-fading media (Vector).

Western Blotting

To evaluate the levels of protein expression (NMDAR, FGFR) in treated and untreated NT2N cells and to further corroborate the localization of aFGF homogenates from harvested cells as well as control and AD cases, cytosolic and particulate fractions were analyzed by Western blot. To verify the obtained bands either a positive control (aFGF, 15 kDa, Accurate; NMDAR1 transfected HEK T 293 cells, courtesy of Dr. H. Schiffer, Salk Institute, La Jolla) or a blocking peptide (FGFR3 control peptide, Santa Cruz) was used. After electrophoreses (12% Tris-Glycine gel for aFGF, 10% Tris-Glycine gel for FGFR3 and NMDAR1) and transfer to nitrocellulose membrane nonspecific binding sides were blocked by PBS BSA 3% for 1 h followed by incubation for 24 h in the primary antibodies (anti aFGF [1:500], anti NMDAR1 (monoclonal IgG, synaptic systems/Göttingen, Germany; 1:1000) [36], anti FGFR3 [1:500]). While 11b (protein A) binds directly to polyclonal antibodies, blots reacted with monoclonal antibodies were incubated in rabbit anti mouse IgG (Accurate) for 2 h prior to incubation in 11b. Immunoreactivity was then detected and quantified using the ImageQuant software ( Molecular Dynamics, Sunnyvale, CA).

Image Analysis and Confocal Microscopy

To semiquantitatively assess levels of aFGF immunoreactivity (IR) per neuron (expressed as optical density (OD)) and the number of aFGF positive neurons, as well as the total number
of neurons a Leica Quantimet 570C equipped with a microdensitometer was used. Estimates of the overall number of neurons per field in the EC were performed in adjacent sections immunolabeled with an antibody against APP. Previous studies have shown that APP is present in virtually all neurons in the brain and that it can be used as a neuronal marker (39, 40). This procedure was favored over more traditional methods because the tissues and approach utilized was relatively similar to the method used for the assessment of aFGF labeled neurons. In 3 different fields (area 0.16 mm²) within CA3, CA1 and EC, aFGF immunolabeled neurons were detected, counted, and OD of all neurons in each region was measured and averaged, as previously described (41). The operator constantly supervised cell detection, ensuring that all pyramidal neurons were recognized and non-neuronal cells were excluded.

The double-immunolabeled sections were viewed with a Zeiss 63X (NA.1.4) objective on a Zeiss Axiosvert 35 microscope with attached laser confocal scanning system MRC 1024 (Bio-Rad, Watford, UK) as described previously (2). Serial optical z-sections (0.2 μm) of the double-immunolabeled structures were collected using the dual channel imaging capability of the confocal microscope. The FITC channel collected the tau positive neurofilbrillary tangles (NFT), NMDAR-α, and FGF-R immunolabeled neurons while the Texas red channel collected the aFGF positive neurons. Each series of sections was scanned through a total of 10 μm. The digitized video images were processed and stored on a 650 Mbyte optical disk for subsequent analysis. In addition, IR of aFGF positive neurons was assessed by evaluating the value of pixels in selected areas. The number of Aβ50 positive NFTs in EC was semiquantitatively assessed using a light microscope (40× lens). In 4 randomly chosen areas within EC2 of all AD and control cases occurring NTF were counted, then averaged.

Cytotoxicity Assay

Culture supernatants were collected 24 h after glutamate exposure and lactate dehydrogenase (LDH) release was determined based on the conversion of tetrazolium salt (INT) into a red formazan product utilizing the reduced form of nicotinamide-adenine dinucleotide (NADH) (42). NADH results from the reduction transforming NAD⁺ and lactate into NADH and pyruvate which is catalyzed by LDH (Cytotox 96, Promega). The assay was performed following the manufacturer’s instructions and the absorbance (A) was then measured at 492 nm. After subtraction of background absorbance values were averaged. The cytotoxicity rate was determined and expressed in percentage \(\left[\frac{A_{\text{abs}} - A_{\text{norm}}}{A_{\text{norm}}}\right] = \text{cytotoxicity [%]}.\)

Cell Survival Assay

Following the collection of the culture media, the number of viable cells was assessed based on the MTT concentration (43). MTT is a tetrazolium salt that is converted into a blue formazan product by viable cells. Cells were incubated in OPTI-MEM I containing 1.5% of the dye solution (CellTiter 96, Promega) for 4 h followed by a stop reaction (2 h). The blue color product was then measured at 570 nm and averaged.

In Situ DNA Fragmentation Assay

In aFGF treated and nontreated NT2N cells, the DNA fragmentation was evaluated as a marker for apoptotic cell death. DNA fragmentation was detected using a modified version of the (TdT)-mediated dUTP-biotin nick-end labeling method of Gavriely (44). Plated cells were washed twice with DNAnse free PBS and then fixed for 20 min with DNAnse free 4% paraformaldehyde. After fixation cells were incubated for 1 h at 37.5°C in a solution containing TdT (0.3 enzyme units/μl) and biotin-16-dUTP (0.02 nM/μl) Boehringer Mannheim) in 1× TdT buffer with Cobalt Chloride. Cells were then washed and incubated in Avidin-HRP (ABC Elite, Vector) for 1 h followed by a reaction with DAB.

Statistics

Comparison of results among the diagnostic groups (AD, Control) was performed using the analysis of variance (ANOVA) with diagnosis as a between subjects factor. Whenever a main effect of the group factor was significant (at least p < 0.05) this effect was followed up by the standard Scheffe’s procedure. For these pairwise comparisons, a p value of less than 5% was accepted as indicating statistical significance. Simple regression analysis was carried out for correlation studies. Calculations were performed using the Statview program package (Abacus Concepts, Berkeley, CA). Values are expressed as mean ± SEM.

RESULTS

Acidic FGF IR in EC and HC is Severely Affected in AD

Acidic FGF IR was observed in pyramidal neurons, non-neuronal cell, and neurites. Acidic FGF IR appeared to be mainly localized in the cytoplasm (Fig. 1a–c). This finding was supported by Western blot analysis showing a positive band at 15 kDa only in the cytosolic (but not particulate) fraction of homogenates of control and AD cases (Fig. 11).

In control cases, aFGF immunoreactive pyramidal neurons were evenly distributed throughout the subiculum while within EC and HC aFGF IR followed the order EC2 < EC layer 6 and CA1 < CA2 < CA3 (Fig. 1a–c). In AD cases a loss of aFGF immunoreactive pyramidal neurons was found while aFGF positive astrocytes in the superficial layer were much more abundant (not shown). Compared with controls, the decrease of aFGF labeled neurons was most severe in EC2 (Figs. 1a, 2a) (F(1, 23) = 60.2, p < 0.0001) revealing an estimated loss of 83.4%, compared with control cases (Fig. 2b). Interestingly, assessment of the total number of EC2-neurons per area revealed only a 70% decrease correlating well with disease progression (r = −0.876, p < 0.0001) (45, 46), while the number of aFGF positive neurons were also dramatically decreased in early stages (Fig. 3). These data might indicate that the decline in the number of aFGF labeled EC2 neurons does not only reflect the neuronal loss occurring in AD but also contributes to the selective vulnerability of EC2 neurons. In contrast, evaluation of the CA1 region of HC (Figs. 1b, 2a) revealed only a moderate decrease (30%, Fig. 2b) in aFGF positive neurons (F(1, 23) = 16, p = 0.0006).
while the slight decline in aFGF expressing neurons in CA3 (16%; Fig. 2b) did not attain significance (Figs. 1c, 2a). Compared with control cases, levels of IR per neuron expressed in optical density (OD) were increased in EC2 (F(1, 17) = 5.4, p = 0.03) (Fig. 4a) and CA1 (F(1, 23) = 10.3, p = 0.004) (Fig. 4a) of AD cases, while no significant increase was observed in CA3 (Figs. 1a–c, 4a).

aFGF IR is Downregulated in Tangle Bearing Neurons

Previous studies have suggested that neurons that develop NFTs are more vulnerable to the degenerative process in AD (47). Therefore, to determine whether levels of aFGF IR in EC2 correlate with NFT formation, double-labeling study for aFGF/tau was performed in selected sections and levels of aFGF IR were assessed by...
Fig. 2. Semiquantitative assessment of number of aFGF immunoreactive neurons in EC2, CA1, and CA3 in control and AD. a: Compared with controls, the number of aFGF positive neurons (per 0.16 sq mm) was dramatically decreased in EC2 in AD. b: Number of aFGF immunoreactive neurons in AD is expressed as percentage of aFGF labeled neurons in controls demonstrating the severe loss of aFGF positive neurons (black) in EC2 (83.4%) compared with CA1 (30%) and CA3 (16%).

Fig. 3. Comparison of the total number of APP immunoreactive neurons with the numbers of aFGF immunoreactive neurons in the EC2. APP was utilized as a neuronal marker to estimate the approximate total number of neurons. Overall, both the control and AD cases had more APP immunoreactive neurons than aFGF immunoreactive neurons. Assessment of the total number of EC2 neurons per area (0.16 sq mm) showed a 70% decrease correlating well with disease progression (Braak stages) (r = -0.876, p < 0.0001), while the number of aFGF positive neurons was also dramatically decreased in early stages (Braak stage 1 and 2).

Fig. 4. Semiquantitative assessment of levels of aFGF IR (a) and FGFR3 IR (b) expressed in OD in EC2, CA1, and CA3: compared with control cases, significantly higher levels of aFGF IR were found in EC2 and CA1 in AD (a), while FGFR3 IR was increased in EC2 only (b). The digits indicate the number of cases that showed aFGF / FGFR3 immunoreactive neurons in the region that was investigated.

Expression of FGF R3 in EC2 in AD is Altered and Correlates with aFGF IR

In control cases, FGFR3 positive neurons were widely and evenly distributed throughout EC, HC, and subiculum (Fig. 1d–f). Within the EC, FGFR3 expressing neurons appeared to be more abundant in layer 5–6 than in EC2 (Fig. 1d). Compared with controls, the number of FGFR3 expressing neurons was decreased in AD. In only 9 out of 15 AD cases FGFR3 immunoreactive neurons were identified in EC2 (Fig. 1d), while the loss of FGFR3 positive neurons was less severe in CA1 (Fig. 1e) and CA3 (Fig. 1f). Analysis of optical density revealed increased levels of FGFR3 IR surviving EC2-neurons (Fig. 4b) compared with controls (F(1, 17) = 7.4, p = 0.015) suggesting a higher expression of FGFR3 in these neurons. To further corroborate possible regulatory effects of aFGF on its receptor in EC2 double-labeling study and correlation analysis were performed. Confocal microscopy of double-immunolabeled section showed a strong colocalization between aFGF and FGFR3 IR (Fig. 5e). Regression analysis revealed positive correlation between these 2 factors (r = 0.532, p = 0.05) suggesting that increased aFGF expression in EC2 neurons results in increased levels of FGFR3 IR (Fig. 7a).

Expression of NMDAR1 is Correlated With aFGF IR in EC2

To investigate the possible interaction between aFGF and NMDAR1 expression in EC2 sections from AD and control cases were single-(Fig. 1h) and double-immunoconjugated for NMDAR1 and NMDAR1/aFGF. Double-labeling showed that aFGF positive neurons also express NMDAR1 (Fig. 5f). Further assessment of levels of NMDAR IR in EC2 followed by statistical analysis showed a negative correlation (r = -0.552, p = 0.008).
AFGF IN AD

Fig. 5. Confocal images of single- and double-immunolabeled sections. Double-labeling with anti aFGF (red) and anti \( \tau \) (green) showed a strong aFGF IR in the cytoplasm of tau-negative neurons (a), while NFT bearing neurons appeared to express lower levels of aFGF (b: tau IR; c: aFGF IR; d: merged). Double-labeling for aFGF (red)/FGFR3 (green) (e) and aFGF (red)/NMDAR1 (green) (f) revealed a strong colocalization between these factors. Omitting one of the primary antibodies (FGFR3 or NMDAR1) demonstrates the authenticity of the reaction (inserts).

![Graph showing correlation analysis](image)

Fig. 6. Correlation analysis revealed a significant inverse correlation between NFT count (per 0.16 sq mm) and aFGF IR (OD) in EC2 in AD (\( r = -0.707, p = 0.02 \)) supporting the hypothesis that aFGF positive neurons are less likely to develop NFT.

between NMDAR expression and aFGF IR in EC2 neurons (Fig. 7b) indicating that aFGF might regulate NMDAR expression.

aFGF Rescues NT2N Cells from Glutamate Induced Cell Death

To support the hypothesis of aFGF playing an important role in neuroprotection in AD effects of aFGF on glutamate induced excitotoxicity were investigated in vitro utilizing mature NT2N cells. About 2.5 weeks after separating non-neuronal cells from neurons, NT2N cells had developed a dense network of neuritic processes (Fig. 8a). Exposure to glutamate led to cell death as shown by DNA fragmentation assay (Fig. 8e) (48), but treatment with aFGF rescued NT2N cells from glutamate-induced cell death as suggested by TUNEL reaction (Fig. 8f). To further assess the neuroprotective effect of aFGF, LDH released by dead cells and MTT converted by viable cells were measured. Correlation analysis between these 2 methods showed a highly significant correlation (\( r = \ldots \))

Fig. 7. A positive correlation between aFGF IR (OD) and FGFR3 IR (OD) in EC2 (r = 0.532, p = 0.05) was found, while aFGF IR (OD) and NMDAR1 IR (OD) were negatively correlated (r = -0.552, p = 0.008).

-0.943; p < 0.0001). Treatment of NT2N cells with aFGF ± H in increasing concentrations prior to glutamate exposure resulted in a highly significant decline of the LDH release (F(8, 4) = 93.5; p < .0001) (Fig. 8a) while the conversion of MTT showed a strong increase (Fig. 8b) (F(8, 4) = 78.1; p < 0.0001). Higher doses of aFGF as well as the substitution of heparin (15, 25, 26, 49) had increasing effects on cell survival (Fig. 9). However, pairwise comparison attained only significance for the comparison between untreated and treated cells (10 ng ± H: p < 0.0001; 25 ng ± H: p < 0.0001; 50 ng ± H: p < 0.0001) as well as between cells treated with low (10 ng) and high (50 ng ± H) concentrations of aFGF (50 ng: p = 0.0014; 50 ng + H: p = 0.0005). In addition, glutamate induced cytotoxicity was assessed relative to the minimal cytotoxicity rate (i.e. cells that were aFGF treated but not exposed to glutamate) versus maximal cell death (i.e. chemically lysed cells) and expressed as a percent value (Fig. 10). In cells treated with 10 ng aFGF±H/ml a glutamate induced cytotoxicity of 6%-10% occurred while treatment with 50 ng aFGF ± H/ml reduced excitotoxic cell death up to 2.4% (compared with 42.5% in untreated cells).

In order to confirm the authenticity of these effects, control experiments were performed by inhibiting NMDAR and aFGF. In the presence of NMDAR blocker MK-801 the neurotoxic effect of glutamate was nearly completely abolished (Figs. 9, 10) while absorption of aFGF resulted in equally high cytotoxicity (Figs. 9, 10) found in nontreated cells. These data indicate that the exposure to glutamate leads to cytotoxic NMDAR stimulation, which can be counteracted by the treatment with aFGF.

NMDAR1 and FGFR3 Expression in Cells after Treatment With aFGF

To semiquantitatively assess the effects of aFGF treatment on the expression of FGFR3 and NMDAR1 Western blot analysis was carried out. As previously described (50), a single band at 125 kDa was identified as FGFR3 protein confirmed by a preabsorption study (no band was detected) (Fig. 8b). Levels of IR were assessed by evaluating the integrated volume of each band (Fig. 8b). A dose dependent increase in IR was found implying that aFGF up regulates the expression of FGFR3 in a dose dependent manner. As previously described (20, 36), NMDAR1 immunoreactive bands were found at 116 kDa and 71 kDa (Fig. 8c). Evaluation of the integrated volume revealed lower values in cells treated with higher doses of aFGF + H compared with nontreated cells indicating a possible regulating effect of aFGF on NMDAR1 expression.

DISCUSSION

Fibroblast growth factors are important molecules involved in mitogenesis, neuritic outgrowth, differentiation, and cell survival (15, 16, 49, 51). Although aFGF seems to be more specific for neuronal tissue than bFGF, for example, most of the studies investigating effects of FGF in the central nervous system concentrated on bFGF. In the present study, patterns of aFGF in EC and HC in patients suffering from AD have been evaluated and evidence has been found that aFGF plays an important role in neuroprotection. While the number of aFGF immuno labeled EC2 neurons was dramatically decreased in AD beyond the expected levels of neuronal loss (46, 47), higher levels of aFGF IR were found in the surviving EC2 neurons in AD compared with controls. Furthermore, in AD, surviving neurons that did not show neurofibrillary alterations displayed strong aFGF IR, while tangle-bearing neurons showed decreased aFGF IR. This might indicate that the loss of aFGF IR might confer increased susceptibility to neurodegeneration and that only those neurons capable of upregulating their aFGF levels might survive. These results also explain why tangle-bearing neurons that are presumably degenerating have low levels of aFGF; while preserved neurons show higher levels of aFGF IR. This is further supported by the in vitro studies showing that treatment of NT2N cells with aFGF prevents glutamate-induced neurotoxicity. These data are consistent with previous studies showing that FGFs have neuroprotective effects against excessive...
Fig. 8. a: 2.5 week old NT2N cell had developed a network of processes (shown after immunolabeling for FGFR3). b: Western blot analysis revealed increased expression of FGFR3 (125kDa) in aFGF treated NT2N cells, while decreased concentration of the 116kDa and 71kDa NMDAR protein (c) was found after treatment with aFGF (HEK: NMDAR transfected HEK cell serving as positive control; abs.: after preabsorption of anti FGFR3 no band at 125 kDa was observed). Assessment of the integrated volume demonstrates the dose effect (0 ng–50 ng). d–f: DNA fragmentation assay illustrates glutamate-induced cytotoxicity in NT2N cells. Compared with control cells (d), exposure to glutamate resulted in extensive cell death as shown by fragmented nuclei (e). Treatment with aFGF could rescue NT2N cell from excitotoxicity (f).

glutamate stimulation (13, 20, 24) and axotomy induced cell death (10). In this context, previous studies (20, 24) have reported that bFGF downregulates the expression of the 71 kDa NMDAR protein. Consistently, a negative correlation between levels of aFGF IR and NMDAR1 expression in AD and controls was found in the present study and confirmed in vitro. Treatment of NT2N cells with aFGF revealed lower levels of the 71 kDa as well as 116 kDa NMDAR1 protein by Western blot analysis. In contrast, significantly higher levels of FGFR3 IR were found in EC2 of AD compared with controls and appeared to be positively correlated with aFGF IR. In fact, aFGF and FGFR3 appeared to be strongly colocalized in EC2 and additional experiments confirmed the interaction between aFGF and FGFR3 that was found in situ. Increased levels of FGFR3 protein were detected in NT2N cells after treatment with aFGF indicating regenerative functions of aFGF on FGFR3 expression. These data are partly consistent with previous results which have reported that stimulation with FGF increases levels of
FGFR mRNA (21), while others found only the intracellular FGF or no FGF being involved in the upregulation of FGFR expression (22, 23).

However, it has been shown that extracellular aFGF is taken up by the cells and transported to the nucleus (26,52). In this context, it has to be considered that not only aFGF, but also FGFR, might be involved in protective mechanisms. Although FGFR3 is most specific for aFGF, other growth factors (e.g. bFGF) also act on this receptor (27). Consequently, it cannot be ruled out that additional factors are involved in the suppression of NMDAR protein. For example, upregulation of FGFR3 by aFGF might lead to increased activity of other FGFs, which results in decreased NMDAR1 expression.

Taking these data into consideration, the present study indicates that aFGF plays an important role in neuroprotection in AD via regulation of receptors (NMDAR, FGFR). Therefore, neurons might respond to neurotoxic stress with accumulation and/or increased expression of aFGF. While high levels of aFGF IR appeared to be associated with neuronal survival, neurons that do not express/accumulate a sufficient amount of aFGF might develop NFTs, lose their ability to synthesize/store aFGF, and finally undergo cell death (47). Consequently, the overall decreased IR for aFGF in EC2 in AD (compared with controls) leads to increased susceptibility to neurotoxicity of the neurons in this region. In fact, a neurotrophic effect of aFGF has been suggested previously (53–56). For example, Tooyama et al (53) found increased levels of aFGF IR in astrocytes in AD implicating a neuroprotective role of aFGF in neurodegeneration. Although aFGF lacks the signal sequence for secretion (15, 27, 50) small amounts of aFGF are released by cells via unusual pathways (26, 51, 57). Therefore, increased levels of aFGF in neurons, as well as astrocytes, might contribute to cell survival in AD.

In summary, the present study provides evidence that aFGF is neuroprotective against glutamate excitotoxicity and implies that loss and/or lack of ability to express aFGF leads to impaired neuroprotective mechanisms (FGFR-, NMDAR-regulation) and, consequently, renders EC2 neurons vulnerable to excitotoxicity and neurodegeneration in AD.

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