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Studies on the Epitope of Neuronal Growth Inhibitory Factor (GIF) with Using of the Specific Antibody

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Human neuronal growth inhibitory factor (GIF), a metalloprotein classified as metallothionein-3, is specifically expressed in mammal central nervous system (CNS). In these Studies the specific antibody to human GIF was prepared and used to search the epitope of human GIF by enzyme-linked immunosorbent assay (ELISA) and sequence comparison. The result of ELISA showed the epitope of human GIF may locate on a octapeptide (EAAEAEAE) in the α-domain of human GIF, and the result of nerve cell culture indicated that the biological activity of GIF may be affected by the specific antibody.

Keywords: Antibody, Domain, Enzyme-linked immunosorbent assay (ELISA), Epitope, Human neuronal growth inhibitory factor (GIF)

Introduction

The pathological pattern of Alzheimer's disease (AD) is characterized by extensive neurodegeneration accompanied by the formation of neurofibrillary tangles and amyloid plaques (Selkoe, 1991). One hypothesis postulated that these alterations are caused by an imbalance of neurotrophic factors (Appel, 1981). Performing in vitro cell culture studies, Uchida *et al.* discovered that protein extract from AD brain increased rat cortical neuron survival more effectively than extract from normal human brain, suggesting that AD brain possesses elevated neurotrophic activity (Uchida *et al.*, 1988). This apparent increase in neurotrophic activity in AD brain was found to be due to the loss of a growth inhibitory factor (GIF), which was subsequently purified from healthy human brains (Uchida *et al.*, 1991).

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The characterization of GIF revealed a metalloprotein of 68 amino acids with its primary structure exhibiting approximately 70% sequence identity with those of mammalian metallothioneins (MTs), including the preserved array of 20 cysteine residues. These features together with molecular biological studies led to its classification as metallothionein-3 (MT-3) (Palmiter *et al.*, 1992; Tsuji *et al.*, 1992). Compared to the amino acid sequence of mammalian MT-1/MT-2, the consensus sequence of MT-3 (GIF) contains two inserts: a single Thr in the N-terminal β-domain and a octapeptide (EAAEAEAE) in the C-terminal α-domain. Additionally, all known GIF sequences contain the conserved C(6)-P-C-P(9) motif, which is absent in all other MTs (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Kobayashi *et al.*, 1993; Pountney *et al.*, 1994; Chen *et al.*, 1996; Kojima *et al.*, 1998).

Despite the high similarities between the primary structures of GIF and other MTs, only GIF but not other MTs exhibits a growth inhibitory activity in neuron cell culture studies (Uchida *et al.*, 1991; Erickson *et al.*, 1994; Bruinink *et al.*, 1998). By now, little is known concerning the reason for this functional difference.

The present work was conducted with the aim of gaining more information on immunology about human GIF. For this purpose, the specific antibody to human GIF was generated. We used enzyme-linked immunosorbent assay (ELISA) and sequence comparison to search the epitope of human GIF. Nerve cell culture system was used to determine the functions of the epitope of human GIF on its inhibitory activity. The structural properties of epitope of GIF likely to be responsible for its biological function are discussed.

Materials and Methods

Expression and purification of antigen Human MT-1, human GIF, human GIF which is binding no metal ion (apoGIF), isolated GIF-α-domain and GIF-β-domain was expressed and purified as described (Wu *et al.*, 1998). The octapeptide (EAAEAEAE) was

chemically synthesized.

Preparation of rabbit anti-human GIF immunoglobulin G (IgG) Antiserum are generated in adult rabbit and purified using a method as described (Robert, 1991), test by ELISA.

Enzyme-linked immunosorbent assay (ELISA) Human MT-1, human GIF, apoGIF, isolated GIF-α-domain and GIF-β-domain, chemically synthesized octapeptide (EAAEAEAE) was used respectively as antigen. Wells of a 96-microtitre plate were coated overnight at 4°C with the antigen at the concentration of 1 μg/ml in CBS buffer (8 mmol/l Na_2CO_3, 42 mmol/l $NaHCO_3$, pH 9.6). The antigen solution was then discarded and residual binding sites were saturated with 1% BSA (bovine serum albumin) in PBS (137 mmol/l NaCl, 3 mmol/l KCl, 10 mmol/l Na_2HPO_4, 2 mmol/l KH_2PO_4, pH 7.4) for 2 h at room temperature. The saturating solution was then discarded and The rabbit anti-human GIF IgG diluted 1 : 2000 in 1% BSA, 0.05% Tween 20 in PBS were added and incubated for 3 h at room temperature. Subsequently the plate was emptied and washed three times with PBS-0.05% Tween 20. The plate was then incubated with the goat anti-rabbit IgG Horseradine Preoxidase-linked (HRP-linked) at the appropriate dilution in PBS, 1% BSA and 0.05% Tween 20 for 2 h at room temperature or overnight at 4°C. After three washes with PBS-Tween 0.05%, the freshly prepared substrate solution (3'3'5'5'-tetramethylbenzidine, TMB) was applied and the OD at 450 nm was read on a automatic microplate reader. Moreover, each test was also taken in duplicate on uncoated wells and the OD developed (always very low) was subtracted from the OD of the respective serum on the antigen-coated wells before calculating the results.

Nerve cell culture system Hippocampus was dissected from neonatal Wistar rats in ice-cold-Ca^{2+}- and Mg^{2+}-free phosphate buffered saline and treated with 0.25% trypsin for 25 min at 37°C. The cells were then dissociated by triturating through the narrow bore of a fire-polished glass pipette. After washing, the single cell suspension was diluted in DMEM supplemented with 10% fetal bovine serum, 5% horse serum and 100 mg/ml glutamine (Gln) to a final concentration of 5×10^5 cells/ml. The cells were seeded (100 μl per well) into 96-well plastic plates (Corning) and cultured in a humidified incubator with 5% CO_2 and 95% air. The medium was replaced with B27- supplemented Neurobasal-A media (1 : 50) and 100 mg/ml Gln after 24-h incubation. To inhibit the proliferation of non-neuronal cells, Cytosine arabinoside was added into the culture to a final concentration of 4 mg/ml at day 3 and incubated for another 48 h. In above nerve cell culture system, addition of GIF at a different concentration in a range of 50-4,050 ng/ml all significantly inhibited hippocampal neuronal survival supplemented with AD brain extract, which was prepared freshly as described before (Erickson *et al.,* 1994). We added the specific antibody at different dilution in a range of 1 : 200-1 : 3200 with GIF (200 ng/ml) and AD brain extract (250 μg protein/ml), determined the hippocampal neuronal survival by MTT assay as described (Liu *et al.,* 2004). Then the inhibitory activity of GIF with its antibody added can be analyzed.

Results and Analysis

Test the specific of the rabbit anti-human GIF IgG To assure the specific of the rabbit anti-human GIF IgG which used as the primary antibody in the subsequent experiments. Human GIF and human MT-1 were used respectively as antigen to compared the immunoreaction of these two proteins. The result showed that the antibody was specific to human GIF and had almost no interaction with human MT-1.

Studies on immunological characters of human GIF Comparison between GIF and apoGIF. To investigate the epitope of GIF is sequence-determined or formation-determined, apoGIF, which known to bind no metal ion and lose its natural formation, was used as antigen as well as human GIF. The results showed the epitope of human GIF may be sequence-determined, and apoGIF exhibited higher immunocompetence than normal GIF, suggesting that GIF may have some advantage to bind the antibody when it loses its natural formation.

Comparison between GIF-α-domain and GIF-β-domain. To further research on the site where the epitope locate, we compared the immunoreactions between GIF-α-domain and GIF-β-domain. The results showed the immunocompetence of GIF focused on its α-domain, while the β-domain possessed almost no immunocompetence.

Since the epitope of human GIF was sequence-determined and probably located on its α-domain, thus we raised a hypothesis that the epitope of GIF maybe located on the sequence in GIF-α-domain which apparently differed from MT-1. The result of sequence comparison (Fig. 1) indicated that the inserted octapeptide (EAAEAEAE) likely to be the epitope site.

Test on the immunocompetence of the octapeptide (EAAEAEAE). To test the former hypothesis we raised, the octapeptide (EAAEAEAE) was chemically synthesized and used as antigen. As shown in Table 1, the octapeptide possessed almost the same immunocompetence as human GIF and GIF-α-domain. Therefore, we concluded that the octapeptide (EAAEAEAE) may be the epitope of the human GIF.

Assays of inhibitory activity of hGIF in nerve cell culture system To examine the veracity of MTT assay which

Human GIF-α-domain	...KKSCCSCCPA ECEKCAKDCV CKGGEAAEAEAEKCSCCQ
Human MT-1-α-domain	...KKSCCSCCPM SCAKCAQGCI CKGAS.....EKCSCCA

Fig. 1. Sequence comparison between human GIF-α-domain and human MT-1-α-domain.

Table 1. Absorbance of ELISA of different antigens including Human GIF. Each antigen was test on four parallel samples, and each absorbance level of ELISA was normalized to 100% to human GIF.

Antigen	Average Absorbance	% to Absorbance of Human GIF
Human GIF	0.053 ± 0.002	100
Human MT-1	0.005 ± 0.001	9.2
ApoGIF	0.059 ± 0.002	114.4
GIF-α-domain	0.056 ± 0.003	106.6
GIF-β-domain	0.010 ± 0.001	19.2
The octapeptide	0.055 ± 0.002	104.4

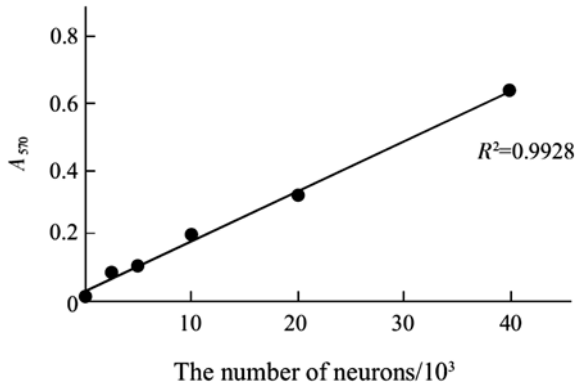

Fig. 2. Absorbance of MTT assay (570 nm) of hippocampal neurons of different concentration.

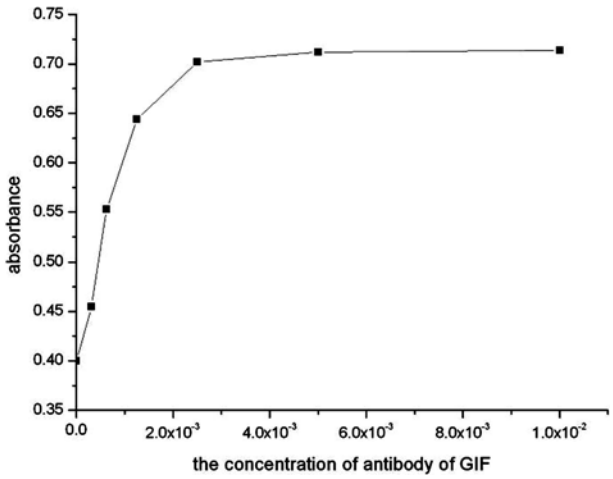

Fig. 3. Absorbance of MTT assay (570 nm) of hippocampal neuronal survival with the antibody of GIF added. The concentrations units of antibody was shown as the dilution of the stock concentration.

determined the hippocampal neuronal survival, we used MTT assay to measure hippocampal neurons of different concentration in a range of 5×10^4-5×10^5cell/well. The result (Fig. 2) showed that the MTT assay could exactly determine the amount of surviving neurons.

The results of MTT assay which to measure the inhibitory activity of GIF with its antibody in different concentration were shown in Fig. 3. These results (Fig. 3) indicated that the specific antibody of GIF severely impaired the inhibitory activity of GIF, and the activity of GIF even can't be observed when the concentration of the antibody is rich enough.

Discussion

This study clearly examined that the epitope of human GIF located on the octapeptide in its α-domain. This result is very unexpected because the epitope of human MT-1 and MT-2 were all established located on their β-domain (Justine, 1991; Nakajima *et al.,* 1991). and most former studies showed that GIF-β-domain was enough for its growth inhibitory activity. Uchida *et al* found out that the 1-23 amino acid residues of GIF or longer peptide segment had the biological activity (Uchida *et al.,* 1995). Hasler *et al.* established that both proline residues in the C(6)-P-C-P(9) motif were necessary to the inhibitory activity (Hasler *et al.,* 2000). All above researches suggested that GIF would bind other factors which necessary to the inhibitory activity on the β-domain.

The results of nerve cell culture may indicate that the inhibitory activity of GIF strongly depended on the spatial conditions. Although the antibody would closely bind GIF on the α-domain, it can prevent GIF to binding other factors on the β-domain, and the interaction between antigen and antibody seems much stronger than which between GIF and the other factors.

As far as we known, few research about the function of GIF-α-domain except metal-binding was reported. Wu *et al* gave some evidences to prove that the hexapeptide (EAAEAE) in GIF may protrude from the molecular surface (Wu *et al.,* 1998). Our present works may reveal some of the biological functions of GIF-α-domain.

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