International Journal of Pharmaceutics 405 (2011) 1–8

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Amelioration of dementia induced by Aβ 22-35 through rectal delivery of undecapeptide-hEGF to mouse brain

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ARTICLE INFO

Article history:
Received 30 August 2010
Received in revised form 19 October 2010
Accepted 11 November 2010
Available online 18 November 2010

Keywords:
Rectal delivery
P11-hEGF
Blood-brain barrier
Aβ 22-35 peptide
Dementia

ABSTRACT

A group of growth factors have been shown to play important roles in amelioration of the malfunction of the neurodegenerative diseases. However, the proteins or polypeptides passing across the blood-brain barrier (BBB) to access the brain parenchyma are relatively few so that it hinders the therapies in clinic. Here a genetically reconstructed fusion peptide of human epidermal growth factor (hEGF) with an undecapeptide YGRKKRRQRRR (P11) was used to investigate the permeability between the cell membrane and the BBB via rectal administration. The efficiency to rescue the Aβ 22-35-induced dementia in mice was assessed after administration of P11-hEGF per rectal. Our results showed that P11-hEGF permeates across not only the 3T3 cell membrane *in vitro*, but also the endothelia of vessels after intravenous injection (IV), and the mucosa of the rectum after per rectal administration. Further results showed that the circulating P11-hEGF allowed penetrating through the blood-brain barrier and then getting into the brain manifesting biological responses. In the animal experiments, treatment with P11-hEGF not only ameliorated the dementia induced by Aβ 22-35 but also rescued the dementia of the aged mice, no matter how it was administrated (IV or per rectal). These results suggest that the rectal non-invasive delivery of the P11 polypeptide-conjugated growth factor is an efficient way for BBB transduction, thus raises the hope of real therapeutic progress against neurodegenerative diseases.

1. Introduction

Some growth factors play important roles in the amelioration of the malfunction of the neurodegenerative diseases (Kuhn et al., 1997; Morrison et al., 1987). However, the proteins and polypeptides passing across the blood-brain barrier (BBB) to access the brain parenchyma are relatively less. It affects the therapy in clinic. In the last decade, lots of investigators have got exciting advance in delivery of bio-drugs (Zhang et al., 2010; Grubb et al., 2010; Matan and Haya, 2009; Zhou et al., 2008; Qu et al., 2008; Rao et al., 2008; Wu et al., 2006; Fu et al., 2004, 2005, 2006). These bio-drugs generally included some protein transduction domains by which enhances the rate and extent of the transportation across BBB without compromising pharmacological properties of the bio-molecules and were administrated by intravenous (IV), subcutaneous (SC) or intracerebroventricular (icv) injections. However, the effectiveness of these bio-drugs still needs to improve and the administration methods by IV, SC and ICV are not perfect for patient who needs a longer period therapy or prophylaxis. A more convenient non-invasive way of administration instead of the frequent injections in clinic is imperative. Human epidermal growth factor (hEGF) can and has been intensively studied.

According to the hEGF and undecapeptide's potential function, here we reconstructed the fusion peptide of hEGF with an undecapeptide YGRKKRRQRRR, named P11-hEGF. We try to use this peptide vector bio-drug to cure the amelioration of the malfunction of the neurodegenerative diseases. The permeability of this polypeptide across the BBB in mice after administration via rectal was measured. Meanwhile, the cellular and biological responses, learning and memory recovery of the Aβ 22-35-induced dementia were investigated after administration of this bio-drug. Our results suggest that rectal non-invasive delivery of the growth factor conjugated with P11 polypeptide, which enhance the ability to traverse the BBB, raises the hope of real therapeutic progress against neurodegenerative diseases.

Abbreviations: DMEM, Dulbecco modified Eagle's medium; FBS, fetal bovine serum; BBB, blood-brain barrier; hEGF, humam epidermal growth factor; P11, undecapeptide (YGRKKRRQRRR); IV, intravenous injection; Aβ 22-35, beta-amyloid peptide 22-35; PTD, protein transduction domain; ELISA, enzyme linked immunosorbent assay; GFAP, glial fibrillary acidic protein; BrdU, 5-bromodeoxyuridine; Amp, ampicillin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DAB, diaminobenzidine tetra hydro-chloride; AUC, area under curve; IPTG, isopropyl β-D-thiogalactoside; OmpA, outer membrane protein A.

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0378-5173/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2010.11.018

2. Materials and methods

2.1. Materials

2.1.1. Reagents

hEGF gene (synthesized by Shanghai Bio-Engineer Co.); pETs, CR MasterMix, DNA markers and BL21 (DE 3) (Tianwei Times Co., China); Restriction endonucleases and T_4 DNA ligase (Promega); PCR primers(Saibaisheng Genotech Co.); *E. coli* TOP10 F and pLPP Vector (Jingmei Biotech Co.); Aβ 22-35, hEGF and IPTG (Sigma); BrdU, GFAP, FITC-labeled goat anti-rabbit antibody, Cy3-labelled goat anti-rabbit antibody, rabbit anti-hEGF polyclonal antibody and horseradish peroxidase labeled goat anti-rabbit IgG (Boster Co.). Other reagents are analytical and chemical pure.

2.1.2. Animals

Mice (Kunming species, age and sex matched) were provided by the Animal Breeding Center affiliated to Academy of Military Medical Sciences in China. Animal studies were approved by the Animal Care and Use Committee, Ministry of Science and Technology, China.

2.1.3. Instruments

The XCS-2 shuttle box and the water maze (Chinese Academy of Military Medical Science); EGF radio-immunological assay kit (The General Hospital of People's Liberation Army); DNA Thermal cycler 480 (PE Co., USA); TGL-16G Desk centrifuge (Anting Medical Instrument Co.); UV-250 spectrophotometer (Shimadzu Corp.); Flow cytometer (BD Co.).

2.2. Methods

2.2.1. Gene construction and expression

The human epidermal growth factor gene was synthesized using the *E. coli* biased-preference nucleotides. The NcoI restriction site and OmpA were added to the 5′-terminus, and the EcoRI site to the 3′-terminus of the P11-hEGF. The recombinant gene OmpA-P11-hEGF was cloned into pETs to construct the pETs-ompA-P11-hEGF plasmid. The secretary expression plasmids were expressed in *E. coli* TOP10 F and BL21 (DE 3) under IPTG induction. The secretary P11-hEGF in the periplasm was extracted and purified by SDS-PAGE (Fig. 1).

The recombinant gene OmpA-P11-hEGF was cloned into pETs to construct the pETs-ompA-P11-hEGF plasmid, and then expressed in *E. coli* BL21 (DE 3) under IPTG induction. The purified P11-hEGF showed a molecular weight of 7.0 kDa.

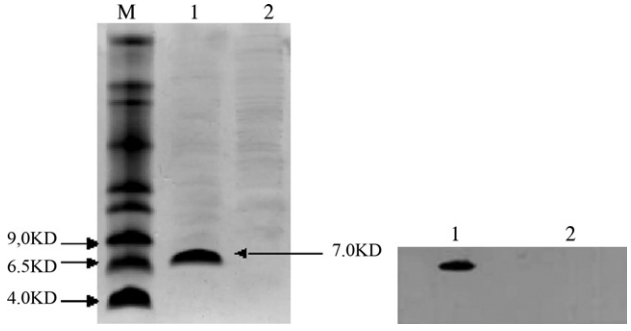

Fig. 1. Profiles of SDS-PAGE and Western blot of the purified P11-hEGF.The recombinant gene OmpA-P11-hEGF was cloned into pETs to construct the pETs-ompA-P11-hEGF plasmid, and then expressed in *E. coli* BL21 (DE 3) under IPTG induction. The SDS-PAGE and western blot profiles of the purified P11-hEGF fusion protein showed a dark single band with a molecular weight of 7.0 kD. (A and B) M, peptide Marker; Lane 1, P11-hEGF; Lane 2, vector control.

2.2.2. Cell membrane permeation in vitro

The cell permeation tests were carried out at 37 and 0–4 °C. Balb/c 3T3 cells (10^4 cells/well) were incubated with hEGF or P11-hEGF (normalized to 10 μg EGF/L) in RPMI 1640 medium containing 10% FBS in a total volume of 2 mL. After incubation under 5% CO_2 for 1 h, the cell membrane permeability was examined by the conventional ELISA using the rabbit anti-hEGF polyclonal antibody (1:200, 4 °C overnight), horseradish peroxidase labeled goat anti-rabbit IgG (1:3000, 4 °C overnight) and DAB staining.

2.2.3. Brain-blood barrier permeation in vivo

The fused P11-hEGF (100 μg in 100 μL) or the commercial hEGF were injected to the mice via the tail veins. The brains of sacrificed mice were quickly removed 4 h later and fixed for 3 days with 4% formaldehyde. Histochemical examination was subsequently performed.

2.2.4. Tissue distribution of P11-hEGF and hEGF after IV or per rectal administration in mice

The mice were divided into 13 groups of 6 and were administered with hEGF (40 μg/mouse) or P11-hEGF (normalized to 40 hEGF μg/mouse) via tail vein or rectum. The control mice were respectively administered with saline or water. Blood and organs were collected 1, 3, 5, 8, 12 and 24 h after administration. The homogenates of organs were prepared (1:10, w/v), and then stored at −20 °C ready for use. The EGF was quantitatively determined using the radio-immunological assay.

2.2.5. Measure of the activity of cell proliferation

The activity of cell proliferation was examined by flow cytometry. The Balb/c 3T3 cells grown at logarithm phase were trypsinized and naturalized with DMEM medium (Hyclone) containing 10% FBS (Hyclone). Aliquots of cells (10^5 cells/well) were incubated for 12 h with hEGF or P11-hEGF (normalized to 1.65×10^{-9} M hEGF/L) in DMEM medium containing 0.4% FBS. The incubated cells were then collected, centrifuged, suspended in 300 μL of pre-cold PBS, and added to 700 μL cold absolute ethanol, and left at −20 °C overnight. The cells were then re-suspended in PBS and treated with 100 μL RNase (1 mg/mL) at 37 °C for 30 min. Stained with 400 μL PI (50 μg/mL) in dark for 10 min, scored with flow cytometer, and analyzed using the SPSS12.0 software.

2.2.6. Nestin, GFAP and BrdU assays

The brains of the mice were immediately removed after the behavior tests. The cryo-slices of the mouse brains were fixed in 1% polyformaldehyde for 5 min. After washing, the rabbit anti-hEGF antibody was added to the slices and kept overnight. The slices were then reacted with the FITC-labeled goat anti-rabbit antibody (for nestin and BrdU assays) and Cy3-labelled goat anti-rabbit antibody (for GFAP assay), respectively for 150 min, and examined under the fluorescent microscope. The effects of P11-EGF on nestin, GFAP and BrdU levels in the fascia dentatus hippocampus were also detected.

2.2.7. Radio-immunological assay (RIA)

EGF quantification was carried out by the EGF radio-immunological assay kit according to the instruction using ^{125}I-EGF as the probe.

2.2.8. Animal grouping and Aβ22-35-induced model mice

The mice (25 ± 2 g, sex-matched) were randomly divided into 7 groups of 8: (1) dementia control mice: ICV Aβ+ IV saline; (2) normal control mice: IV saline; (3) shame-operated control: ICV saline; (4) P11-hEGF mice: ICV Aβ+ IV P11-hEGF; (5) P11-hEGF mice: ICV Aβ+ per rectal P11-hEGF; (6) hEGF mice: ICV Aβ+ IV hEGF; (7) hEGF mice: ICV Aβ+ per rectal hEGF.

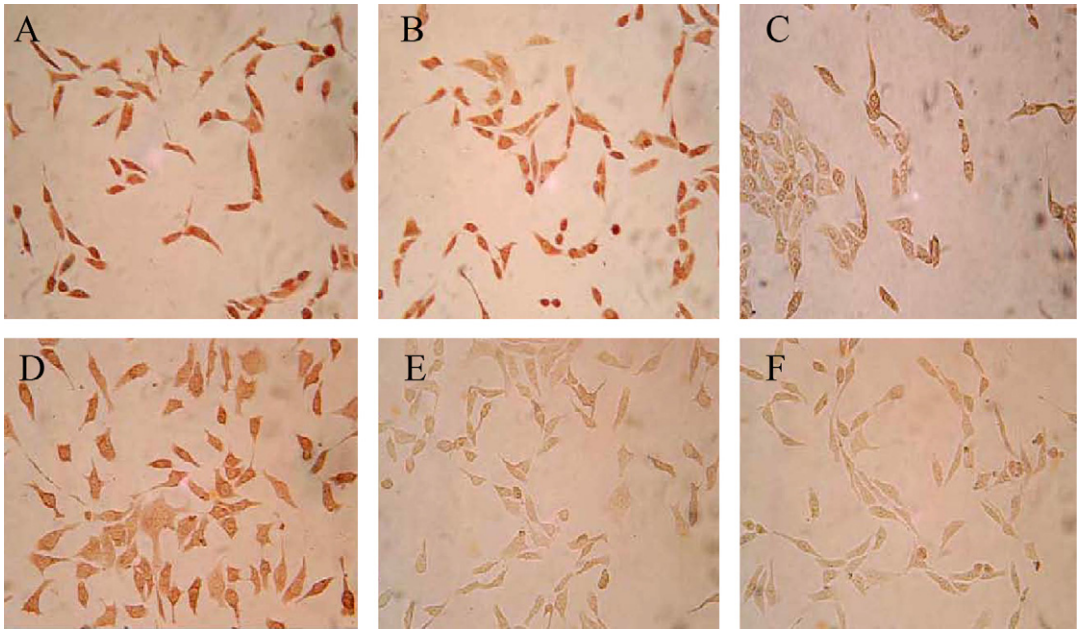

Fig. 2. 3T3 cell permeation of P11-hEGF and hEGF.The cell permeation tests were carried out at 37 and 0–4 °C. Balb/c 3T3 cells (10^4 cells/well) were incubated with hEGF or P11-hEGF (normalized to 10 μg EGF/L) in RPMI 1640 medium containing 10% FBS in a total volume of 2 mL. After incubation under 5% CO_2 for 1 h, the cell membrane permeability was examined by the conventional ELISA using the rabbit anti-hEGF polyclonal antibody (1:200, 4 °C overnight), horseradish peroxidase labeled goat anti-rabbit IgG (1:3000, 4 °C overnight) and DAB staining. (A and D) P11-hEGF; (B and E) hEGF (Sigma); (C and F) control. (A–C) 37 °C; (D–F) 0–4 °C.

The Aβ 22-35 (AEDVGSNKGANGLM) was prepared in saline (1 mg/mL), sealed in ampoule, and transformed into the "aggregated phase" at 37 °C for 96 h (Maurice et al., 1996). The aggregated Aβ 22-35 (4 nmol in 4 μL) was intra-cerebroventricularly (ICV) injected to mice according to the procedure established by Laursen and Balknap (1986). The shame operated control mice received the same volumes of saline instead. The memory and cognition tests were carried out at day 12 after the Aβ 22-35 injection, and the hEGF (4 μg/d) or P11-hEGF (normalized to 4 μg hEGF/d) were then administered via IV or per rectal for 10 consecutive days to the Aβ 22-35-induced dementia mice.

2.2.9. Water maze tests

The behavior of mice was performed in a round (1 m in diameter) water maze (23–25 °C, water depth 31 cm) with a platform (9 cm in diameter, 1 cm lower than the water surface). The swimming tracts were automatically monitored and recorded by the camera and computer. Mice with latencies (the swimming time from the starting point to the platform) higher than 90 s in the screening tests were used as the model mice.

2.2.10. Shuttle box tests

The shuttle box was made of perspex walls and metal grid-floor. On each ends of the box, there was a light beam which would switch on the electricity and evoked a 3-s buzzing following up a 5-s shock on feet of the mouse as soon as the light beam had been blocked by the body. In the trail, at least 10 rounds were performed for each mouse with 23-s time intervals. The sum of the stimulation time was automatically recorded for assessment of the learning and memory abilities for each mouse. The time periods of the accumulative total of all the Aβ 22-35-induced dementia mice were longer than 20 s.

2.2.11. Statistical analysis

Data were pooled from three independent experiments (mean ± SEM). The statistical significance was compared using the student's *t*-test.

3. Results

3.1. Cell permeation of P11-hEGF and hEGF

Experiments were carried out at 37 °C and 0–4 °C *in vitro* by ELISA to examine whether there was any difference in membrane permeation between P11-hEGF and hEGF. The results showed that both P11-hEGF (Fig. 2A) and hEGF (Fig. 2B) could well penetrate through the cellular membranes of the Balb/c 3T3 cells at 37 °C. However, at 0–4 °C, the cells incubating with P11-hEGF showed intensive color (Fig. 2D), while those incubated with hEGF (Fig. 2E) showed faint color as what happened in the control (Fig. 2F). It suggests that the permeation of hEGF across cell membrane is temperature-dependent, but that of P11-hEGF is temperature-independent. The P11-hEGF is much powerful in penetration of cell membranes at both temperatures.

3.2. Difference between P11-hEGF and hEGF in permeation through blood-brain barrier

Experiments were carried out using the histochemical assay to investigate the permeation of P11-hEGF and hEGF through the blood-brain barrier *in vivo*. The mouse brain tissues were collected 4 h after IV injection with P11-hEGF or hEGF. The results showed that the neurons of the mice injected with P11-hEGF showed deep brown color (Fig. 3A), whereas those of the mice injected with hEGF showed very light color (Fig. 3B) similar to the control mice received the same volume of saline (Fig. 3C). It implies that the IV injected with P11-hEGF can traverse the BBB and enter into the brain tissue whereas the hEGF in the circulating blood cannot span over the BBB membranes.

3.3. Distribution of P11-hEGF and hEGF in vivo

The distribution experiments were performed in mice IV injected with hEGF (40 μg hEGF/mouse) or P11-hEGF (normalized to 40 μg hEGF/mouse). The animals were killed at various time intervals after injection, and the organs were immedi-

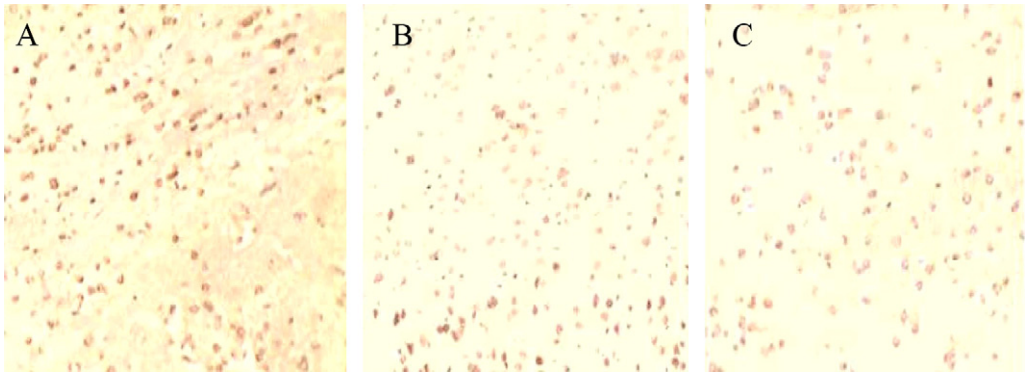

Fig. 3. Permeation of P11-hEGF across the blood-brain barrier of mice.100 μl of P11-hEGF or hEGF (normalized to 1 μghEGF/μl) was injected via the tail veins to the mice. The brains were removed 4 h later, fixed in 4% formaldehyde for 3 days and brain slices were made in the conventional way. (A) P11-hEGF; (B) hEGF (Sigma); (C) control.

ately removed. EGF in various tissue homogenates (1:10, w/v) were quantified using the radio-immunological assay. The results showed that the EGF concentrations in all tissues (heart, liver, spleen, lung, kidney and stomach) except the brain reached their peak at 1 h after injection. The peak concentrations in case of P11-hEGF were about 2-fold higher than those of hEGF (Fig. 4A and B). It is noteworthy that the P11-hEGF entering into and removing from the brain tissues is rather slower than those of the other tissues. The brain concentration of EGF in case of P11-hEGF reached the peak (ca. 52 ng/g tissue) 5 h after IV injection and declined to the base level (the endogenous level of mouse brain, ca.17 ng/g tissue, Fig. 4A) after 25 h. It is also notable that, in sharp contrast to P11-hEGF, the concentration of EGF in the brains of mice IV injected with hEGF did not change compared to the endogenous level (Fig. 4B) during the period of examined 25 h. It implies that the IV injection with P11-hEGF can effectively penetrate through all the membranes examined including the BBB, whereas the hEGF can only permeate through the membranes of the blood vessels and the cells of the visceral organs. Thus we conclude that the P11-hEGF is much better than hEGF *per se* in usage for elevation of the hEGF level in the brain.

In order to investigate whether P11-hEGF can penetrate through the BBB entering the brain via a non-invasive way instead of the IV or ICV administration, the P11-hEGF (normalized to 40 μg hEGF/mouse) was administered via the rectum of the mice. EGF in various tissue homogenates were also quantified using the radio-immunological method. The results showed that after per rectal P11-hEGF administration, EGF concentrations in the visceral organs reached the peaks after 5 h and went back to their base levels after 25 h (Fig. 4C). As expected, in case of per rectal hEGF administration, the EGF levels in each organ did not change during the period of the examined 25 h (Fig. 4D). The result implies that the P11-hEGF can be effectively uptake through the rectum but needs a longer time (versus the IV injection) to traverse the rectal membrane. On the contrary the hEGF is hard to be uptake via this way (Fig. 4D).

3.4. Bio-availabilities of P11-hEGF and hEGF

Since the P11-hEGF can be successfully delivered into the brain after administration per rectal, it is of interest to examine the bioavailability of both P11-hEGF and hEGF in mice. The bioavailability

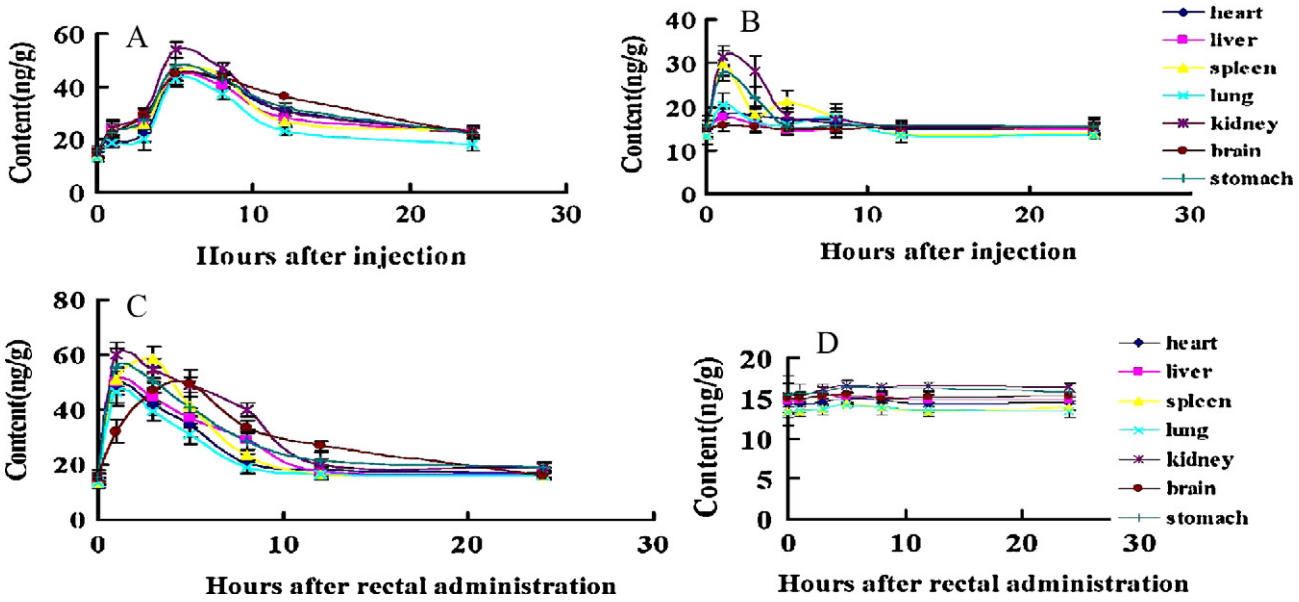

Fig. 4. Distribution of P11-hEGF and hEGF after IV injection or per rectum in mice ($n = 6$).P11-hEGF or hEGF (normalized to 40 μg hEGF) were IV injected to the mice. The animals were killed at various time intervals after injection, and the organs (brain, heart, liver, spleen, lung, kidney and stomach) were immediately removed. Tissue homogenates (1:10, w/v) were prepared and stored at −20 °C. The contents of EGF in various tissue homogenates were quantitatively determined by the radio-immunological method. (A and C) P 11-hEGF; (B and D) hEGF (Sigma).

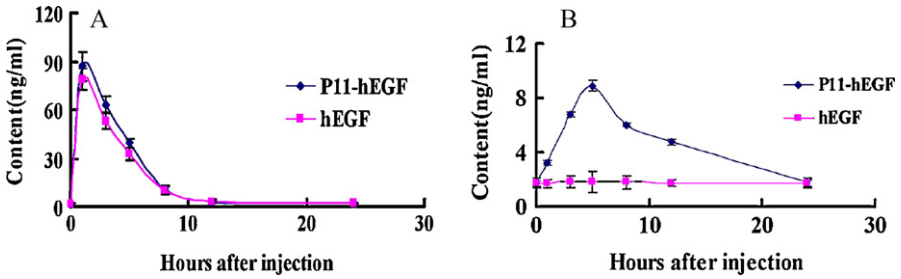

Fig. 5. Blood concentration of P11-hEGF or hEGF after administration via IV or per rectum ($n = 6$).The bioavailability is expressed in terms of the AUC ratio of the blood concentration-time lapse curves after per rectal and IV administrations. (A) IV injection; (B) per rectum administration.

is determined in terms of the AUC ratio of the blood concentration-time-lapse curves after per rectal or IV administrations. The results showed that, after IV injection, the blood levels of P11-hEGF and hEGF in mice quickly reached to their peaks, and removed out from the circulating blood within 10 h (Fig. 5A). However, the EGF level in case of P11-hEGF reached its peak 5 h after administration per rectum, and then gradually decreased 20 h later (Fig. 5B) much slower than those of IV administration (Fig. 5A and B). The maximum EGF concentration in mice after administered with P11-hEGF per rectal (9 ng/mL) was only one tenth of those of administered via IV (90 ng/mL). The rectal administered hEGF did not immerge in the blood circulation at all, only the endogenous EGF appeared in the circulating blood (2 ng/mL) (Fig. 5B).

3.5. Promotion of cell proliferation by P11-hEGF and hEGF

Based on the understanding about the characteristics of the hEGF and P11-hEGF in permeation of BBB and other membranes, the biological functions of and the differences between hEGF and P11-hEGF were further investigated. First, we examined the influence of hEGF or P11-hEGF on the cell proliferation. The Balb/c 3T3 cells at logarithm phase were incubated with P11-hEGF or hEGF at 37 °C for 30 min, and then assayed by flow cytometry. The cell proliferation index (CPI) was used as a measure of the extent of the cell proliferation in terms of the percentage of the sum of cells at S, G2 and M phases to the total number of cells in the whole cell cycle (G1 + S + G2 + M).

G1 pre-synthesis phase;
S DNA synthesis phase;
G2 pre-mitosis phase;
M cell mitosis phase.

The results showed that both the P11-hEGF and hEGF significantly promoted the proliferation of the 3T3 cells, compared to the control ($p < 0.001$, Fig. 6), no significant difference was observed between the P11-hEGF and hEGF treatment.

3.6. Rescue of Aβ22-35-induced learning and memory deficits in mice

The therapeutic actions of P11-hEGF and hEGF to the learning and memory deficits in mice were investigated by the water maze and shuttle box tests.

Water maze test: The therapeutic effects of P11-hEGF and hEGF were tested in the Aβ22-35-induced dementia mice. Twelve days after ICV injection with Aβ22-35, the hEGF (4 μg/d) or P11-hEGF (equivalent to 4 μg hEGF/d) were administered through IV or per rectal to mice for ten consecutive days. The results showed that P11-hEGF administered through either IV or per rectum could significantly shorten the latencies in the Aβ22-35-induced dementia mice as compared with those of the Aβ22-35-model mice ($p < 0.01$, Fig. 7A and C). On the contrary treatment with hEGF did not show any therapeutic effect on the Aβ 22-35-model mice ($p > 0.05$, Fig. 7A and C). Interestingly, the same therapeutic effect with P11-hEGF also happened in the naturally aged mice (Fig. 7B and D).

Shuttle box test: The protocol of therapeutic treatments for Aβ 22-35-induced dementia mice with P11-hEGF or hEGF were same as described in the water maze test. The results showed that treatments with P11-hEGF were therapeutically effective either in Aβ

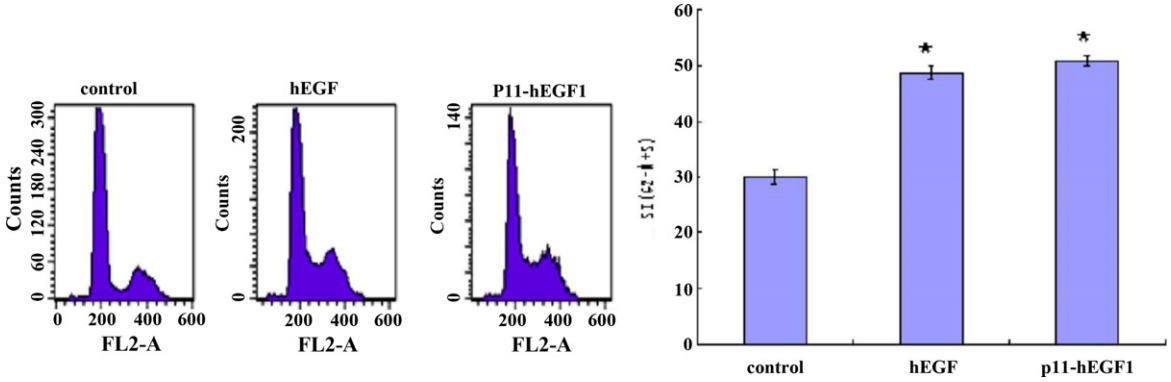

Fig. 6. Promotion of cell proliferation by P11-hEGF or hEGF ($n = 3$).P11-hEGF or hEGF (1.65×10^{-9} mol/L) in DMEM medium containing 0.4% FBS was added to Balb/c cells (1×10^5 cells/well) and cultivated for 12 h. The cells were collected and resuspended in 300 μL PBS containing 5% FBS. Cold absolute alcohol (700 μL) was then added to the cell suspension and stored at −20 °C for 24 h. After centrifugation (3000 rpm, 1 min) the cells were suspendended again in PBS and reacted with RNase (1 mg/mL, 100 μL) at 37 °C for 30 min PI (50 μg/mL, 400 μL) was added for cell staining in the dark for 10 min. The proliferation activity was examined by flow cytometry, and analyzed using the SPSS12.0 solftware. The cell proliferation index was calculated by the equation: SI (%) = G2 + M + S.

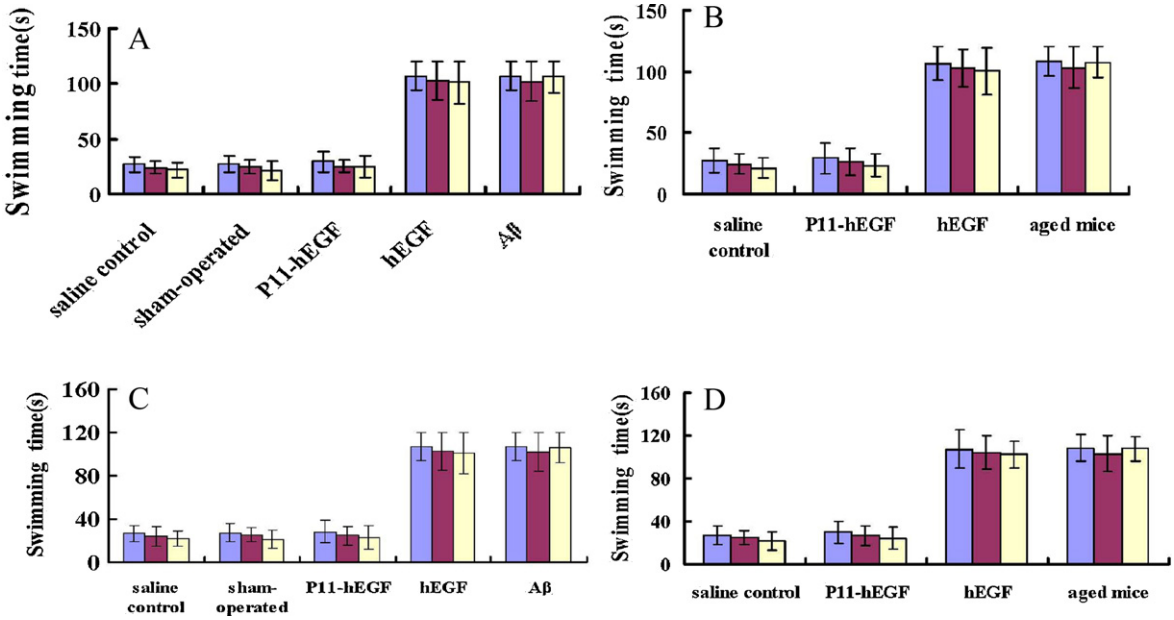

Fig. 7. Therapeutic effects of P11-hEGF against Aβ 22-35-induced dementia in mice ($n = 8$).The normal mice were trained twice a day for 5 consecutive days to acclimatize the water maze. The swimming time from the starting point of the maze to the platform in the water was expressed as latency. The mice with latency less than 2 min were chosen in the Aβ 22-35-induced dementia moulding. Twelve days after icv injection of Aβ 22-35, the hEGF or P11-hEGF (normalized to 4 μg hEGF/d) was IV or per rectal administered to mice for 10 consecutive days. (A and B) via tail vein; (C and D) via rectum; (A and C) Aβ-model mice; (B and D) aged mice.

22-35-induced dementia mice (Fig. 8A and C) or in the aged mice ($p < 0.01$, Fig. 8B and D) no matter how to administrate via IV or per rectum, whereas the hEGF did not show any effect ($p > 0.05$, Fig. 8).

3.7. *Impacts of P11-hEGF therapy on nestin, GFAP expressions and BrdU incorporation in Aβ 22-35-induced dementia mice*

The therapeutic effects of P11-hEGF on the Aβ 22-35-induced dementia mice were also examined refer to some bio-markers of the stem cells. The brains of mice were collected after the behavior tests (water maze and shuttle box tests) and sacrificed, and the cryo-slices of the brains were prepared. The results showed that the expression of nestin in the hippocampus gyri of the Aβ 22-35-induced dementia mice (Fig. 9, top A) was obviously lower than that in the sham treated mice (Fig. 9, top C). After treatment with the P11-hEGF the expression of nestin significantly increased (Fig. 9, top B). GFAP in the hippocampus gyri of the Aβ 22-35-induced dementia mice was higher (Fig. 9 middle A) than that in the normal mice (Fig. 9, middle C). After treatment with the P11-hEGF, the GAFP in gyri obviously decreased (Fig. 9, middle B). BrdU incorporation decreased in the Aβ 22-35-induced dementia mice (Fig. 9, bottom A) in comparison with the normal mice (Fig. 9, bottom C). The incorporation rate of BrdU increased again after treatment with P11-hEGF (Fig. 9, bottom B). Collectively, it implies that P11-hEGF plays an important role in promoting the proliferation of the neuronal stem cells in the central nerve system.

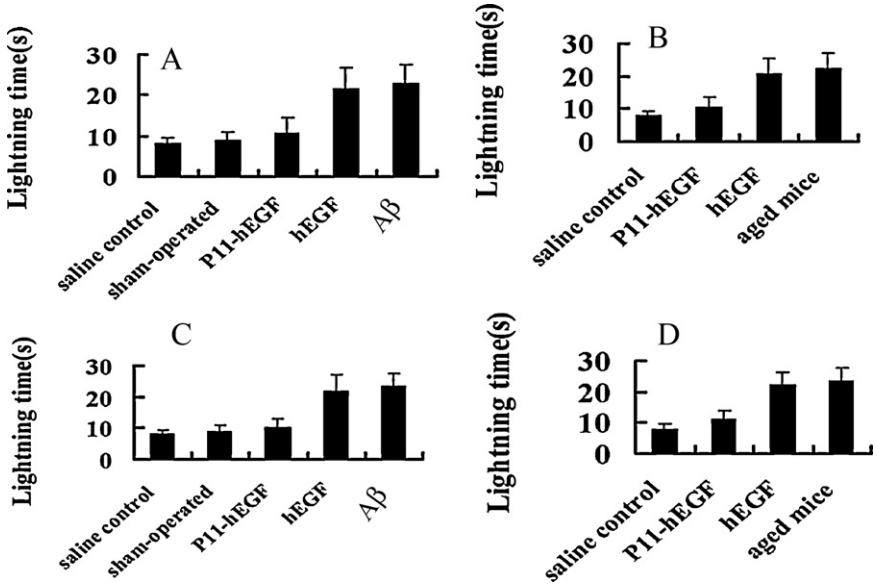

Fig. 8. Therapeutic effects of P11-hEGF against Aβ 22-35-induced dementia in mice ($n = 8$).The shuttle box test (buzzing 3 s, electric foot-shock 5 s) was used in the experiments. Every mouse had to perform 10 rounds with 23-s time intervals in each trial. The learning and memory abilities of each mouse were assessed by the time periods of avoidance followed the electric shock. (A and B) via tail vein; (C and D) via rectum; (A and C) AD-model mice; (B and D) aged mice.

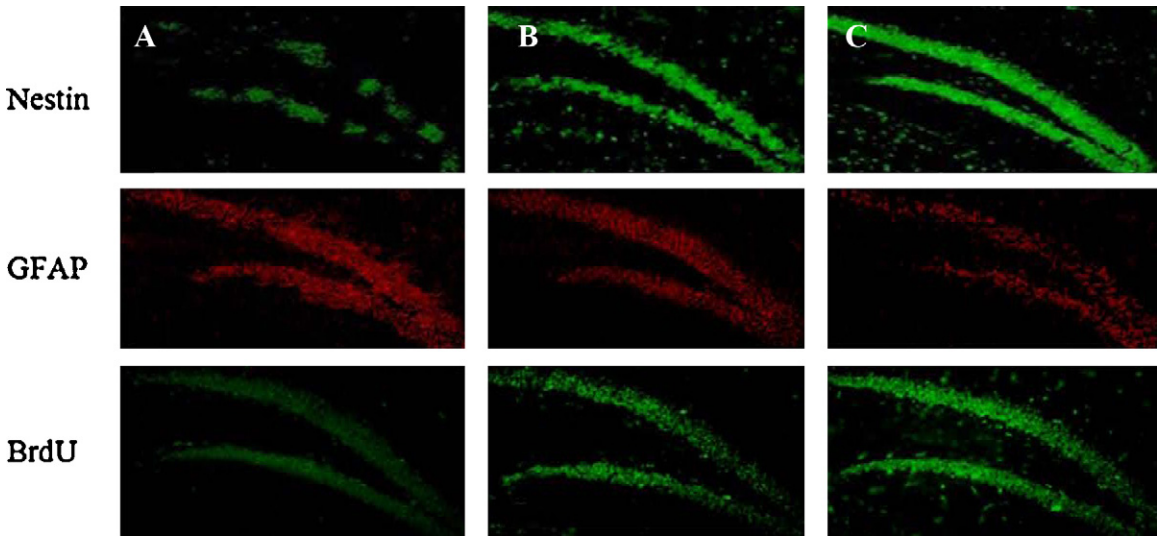

Fig. 9. Impact of P11-hEGF on nestin and GFAP expressions and on incorporation of BrdU in hippocampus gyrus in Aβ 22-35-induced dementia mice (100×).The mouse brains were removed immediately after the behavior tests. The cryo-slices of the fascia dentatus hippocampus were examined by immunohistofluorometry. The rabbit anti-hEGF antibody was used as the first antibody, and the FITC-labeled goat anti-rabbit antibody (for nestin and BrdU assays) and Cy3-labelled goat anti-rabbit antibody (for GFAP assay) were used respectively as the second antibodies. Top: nestin; middle: GFAP; bottom: BrdU. A, AD-model mice; B, P11-hEGF treated AD-model mice; C, sham-operated mice.

4. Discussion

Whether the peripherally administered exogenous peptides can permeate through the BBB has been in dispute for a long time. Different studies showed different results or contrary conclusions. Recently, owing to the large amounts of reports about the membrane permeation issues, it seems to be generally acknowledged that peripheral administration of some exogenous peptides to animals are able to cross the BBB and reach to a certain concentration by different transport pathways including the receptor-mediated endocytosis, lipid craft-mediated pinocytosis, simple diffusion, or via the leaky sites on the BBB and other unknown pathway, if any. It has been demonstrated that many peptides after IV injection appeared in the brain parenchyma with their intact forms, and exhibited various biological responses (Pan et al., 2005, 2007, 2008; Pulford and Ishii, 2001; Reinhardt and Bondy, 1994; Gutierrez et al., 1993; Plata-Salaman, 1988; Panaretto et al., 1982). Although the peptides permeating into the brain is weak, it is significant to evoke certain biological responses. For example, only 0.3% of the IV injected nesfatin-1 (Pan et al., 2007) or less than one hundredth of the IV injected gonadotropin (Yang et al., 2007) permeating into the brain was efficient to induce the biological responses. It seems that the peripherally administered peptides can indeed pass through the BBB and influence the brain functions ameliorating the pathological status. However, some studies did not show the peripherally injected peptides in the brain and the biological response. It may ascribe to the low sensitivity and limitation of the identification methods, or the trace amounts of peptides or proteins (e.g. the enzyme preparations) permeating into the bodies that were not enough to evoke the biological response as what happened in the cases of cytokines.

In clinical practice, there is an unavoidable issue that frequent ICV or even IV and SC injections in purposes of prophylaxis or therapy are not amenable to patients in comparison with the non-invasive way, for example, the sublingual ingestion and the nasal spray. The administration of the P11-hEGF via sublingual ingestion or nasal spray is the desirable choice. However, sublingual or nasal administration is practically difficult in animal models, so the rectum route was adopted as the first step in our research. Our results showed that hEGF *per se* was able to pass through the cell membranes *in vitro* and blood vessels *in vivo*, but it cannot traverse the rectum epithelium and the blood-brain barrier. However, the P11-hEGF permeates through not only the 3T3 cell membrane *in vitro*, but also the endothelia of vessels after IV injection, and the mucosa of the rectum after per rectal administration. It indicates that in case of the P11-hEGF, two routes might be involved to step over the cell membrane, the endocytosis pathway and the P11-transduction pathway.

The further results showed that the circulating P11-hEGF indeed permeated through the BBB into the brain and manifested its biological responses. Animal model experiments exhibited that the treatment with P11-hEGF not only ameliorated the dementia induced by Aβ 22-35 but also rescued the dementia of the aged mice, no matter how it was administrated, via IV or per rectum. The reason why P11-hEGF is much efficient in membrane permeation and in therapy of the malfunctions of brains in the aged and the Aβ 22-35 induced dementia mice than hEGF *per se*. We supposed that the undecapeptide (P11) moiety in the fused molecule plays an important role. It cargos the exogenous polypeptides passing through cell membranes by virtue of the route of receptor-mediated endocytosis and also the route of lipid craft-mediated pinocytosis (Kaplan et al., 2005). It reasonably anticipates that the oral and nasal mucous membranes will be better permeable for many kinds of P11-conjugates versus the rectal mucosa, since the oral and the nasal mucous membranes are thinner and much tender than the rectal one.

5. Conclusion

Our experimental results demonstrated that P11-hEGF permeation through the biomembranes is much efficient than hEGF *per se*. The P11-hEGF administered via rectum can pass through the rectal membrane and the blood-brain barrier to the central nervous system. The symptom of Aβ 22-35-induced and the aged dementia in mice was relieved. It implied that the pharmacological delivery of P11-hEGF (and perhaps other bio-drugs) by a non-invasive peripheral route (e.g. per rectal, nasal or sublingual) to rescue the neurodegenerative dementia (and perhaps other diseases) is potentially feasible and efficient. The rectal delivery of P11-hEGF is a real progress in therapeutic and prophylactic treatments of the neurodegenerative disorders.

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