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Research Article

Kringle 1-5 Induces Neurite Outgrowth and Enhances Dopaminergic Neuronal Survival in Mesencephalic Cultures

Yi-Lo Lin[1,2#], May-Jywan Tsai[2#], Dann-Ying Liou[2], Chen-Jei Hong[2], Kai-Ting Chang[2,3], Chi-Te Lin[2,4], Yang-Hsin Shih[5], Henrich Cheng[2,3,6*], and Ming-Chao Huang[2,4,7,8,*]

[1]*Graduate Institute of Veterinary Pathobiology, College of Veterinary Medicine, National Chung Hsing University, Taiwan*
[2]*Neural Regeneration Laboratory, Neurological Institute, Taipei Veterans General Hospital, Taiwan*
[3]*Department and Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taiwan*
[4]*Departments of Nursing, Central Taiwan University of Science and Technology, Taiwan*
[5]*Neurological Institutes, Taipei Veterans General Hospital, Taiwan*
[6]*Divisions of Neural Regeneration and Repair, Neurological Institute, Taipei Veterans General Hospital, Taiwan*
[7]*Division of Pediatric Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, Taiwan*
[8]*Schools of Medicine, Taipei Medical University, Taiwan*
[#]*Equal contribution*

***Corresponding authors**
Ming-Chao Huang, Division of Pediatric Neurosurgery, Neurological Institute, Taipei Veterans General Hospital, No. 201, Shi-Pai Road, Sec. 2, Taipei, 112, Taiwan, Tel: 886-2-2875-7491; Fax: 886-2-2875-7588; E-mail: mchuang@vghtpe.gov.tw
Henrich Cheng, Division of Neural Regeneration, Neurological Institute, Taipei Veterans General Hospital, No. 322, Shi-Pai Road, Sec. 2, Taipei 112, Taiwan, Tel: 886-2-2875- 7718; Fax: 886-2-2875-7588; Email: hc_cheng@vghtpe.gov.tw

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Abstract

Kringle 1-5 (K1-5) is a proteolytic enzyme fragment of plasminogen. K1-5 was proved to be effective on reducing vascular formation and limiting the tumor growth in a high-grade malignant glioma. However, as far as we know, there was no related research of K1-5 on neurons. In this study, the effect of K1-5 to the brain tissue was conducted in cultured neurons obtained from embryonic cortex and mesencephalon. Results demonstrated that K1-5 increased neurite connection in cultures of both regions as evidence by β III tubulin staining. Especially in the mesencephalic culture, K1-5 increased the number of TH positive dopaminergic neurons, stimulated its neurite outgrowth and protected MPP+ toxicity. Result from western blot analysis demonstrated that the phosphorylation levels of Akt and PKC were increased by K1-5 in mesencephalic culture. It is concluded that K1-5 has general neuroprotective effect that might be mediated through PKC and Akt phosphorylation in mesencephalic neurons.

ABBREVIATIONS

PGN: Fragments of Plasminogen; K1-5: Kringle 1-5; TH: Tyrosine Hydroxylase; Mes: Mesencephalon; CNS: Central Nervous System; MPP^+: 1-Methyl-4-Phenylpyridinium

INTRODUCTION

Fragments of plasminogen (Pgn) have been classically associated with anti-angiogenesis. For example, some proteolytic fragments of Pgn such as angiostatin (Kringle 1-3 or 1-4) and Kringle 1-5 are known for its potency on inhibition of endothelial cell proliferation [1,2]. Its anti angiogenic ability in tumor has also been reported in murine hepatocellular carcinomas [3] and human breast tumors in nude mice [4]. In our recent study, we proved that Kringle 1-5 was effective on reducing vascular formation and limiting the tumor growth in a high-grade malignant glioma [5].

Besides the involvement with vascular process, recent researches have shown the participation of Pgn in neurologic functions. Hydrocephalus has been recognized in Pgn-deficient mice [6]. Patients with severe type I Pgn deficiency exhibit symmetric internal hydrocephalus with a Dandy-walker malformation, hypoplasia of the cerebellum and a hypoplastic corpus callosum [7]. In plasminogen knockout mice, there was a faster transmissible spongiform encephalopathies symptoms and subsequent death for scrapie infected animals, suggesting plasminogen has a neuroprotective effect [8]. These results suggest a role for plasminogen in neuritogenesis.

Pgn contains five kringle structures in total [9]. K1-5 used in this study contains the first four disulfide-linked structures (K1-4) and most of the K5 structure of Pgn. The involvements of Pgn in neurologic aspects allow us to speculate that K1-5 might have significant effects on neurons. In this study, we provided evidence that similar to Pgn, K1-5 induced neurite outgrowth and enhanced neuronal survival in cortical and mesencephalic cultures. It had general neuroprotection effect that might be mediated through multiple signal transduction pathways in mesencephalic neurons.

MATERIALS AND METHODS

Reagents and animals

Cultured media and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Culture plates or dishes were obtained from Corning Life Sciences (Acton, MA). 1-Methyl-4-phenylpyridinium iodide (RBI MPP+) was from Sigma. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich Co. Adult female Sprague-Dawley rats were maintained according to the guidelines established in the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, USA. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Taipei Veterans General Hospital, Taiwan.

Mixed Neuronal/glial culture and treatment

Mixed neuronal/glial cell cultures were prepared from cortex and mesencephalon of embryonic Sprague-Dawley rat brains at gestation day 14-16, as previously described [10-12]. Briefly, tissues were dissociated with mixtures of papain/protease / deoxyribonuclease I (0.1% / 0.1% / 0.03%) and plated onto poly-lysine coated 24-well plates at a density of 2×10^5 cells/cm^2. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% FBS and incubated at 37°C in a water-saturated atmosphere of 5% CO_2/95% air. Beside βIII (+) neuronal cells, the cultures also consist of GFAP (+) astroglia and ED1 (+) microglia as shown in our previous articles [5]. Second day after initial cell seeding, culture were refilled with DMEM supplemented with N2 supplement (Gibco, for serum-free condition) and treated with K1-5 (150 ng/ml) in the presence or absence of MPP^+, an oxidation product of dopamine neurotoxin MPTP [13,14]. Human plasminogen K1-5 was kindly provided by Professor Y. Cao (Karolinska Institute, Sweden). The dosage of K1-5 was selected according to our previous study [5]. Two days after treatment, cells were fixed for morphological assay and culture medium was collected for lactate dehydrogenase (LDH) assay. A commercial kit (CellTiter 96 Aqueous; Promega Corporation) was used to detect the release of LDH in culture medium according to the manufacturer's instructions.

Immunochemical analysis

At definite time, cells were washed with PBS and then fixed in 4% paraformaldehyde solution for 20 minutes. After triton X-100 permeabilization, cells were incubated with primary antibodies: rabbit βIII tubulin (1: 500, Covance, Berkeley, CA) and mouse tyrosine hydroxylase (TH, a dopaminergic neuron marker) (1: 100, Chemicon). The secondary antibody FITC donkey anti-rabbit and Cy3 donkey anti-mouse (Jackson Immuno-Research, West Grove, PA) were used. Images were visualized with a Zeiss fluorescent microscopy (Axioskop 2, Carl Zeiss). To analyze the neurite outgrowth, the length of process was measured with Image J and divided by the cell number. A minimum of 30 cells per well was counted in duplicate cultures. The total number of TH-positive neurons in each well was counted in duplicate cultures. Experiments were repeated 3-4 times.

Western blot analysis

Protein expression levels were quantified by western blot analysis. Cells from mesencephalic culture were washed twice with phosphate buffered saline (PBS) and solubilized in lysis buffer containing PBS, 1% NP-40, and protease inhibitor kit (BM, Mannheim, Germany). Protein concentration of the resultant lysate was determined using the method of Bradford (Bio-Rad protein assay, Bio-Rad Laboratories Ltd, Montreal, Quebec). Equal amount of proteins were loaded and separated using 8-12% gels (SDS-PAGE) as previously described [11]. After gel separation, protein bands were transferred to PVDF membrane (Millipore Immobilon-p, IPVH00010). The resulted membrane was probed overnight at 4°C with primary antibodies at the appropriate dilution, and then incubated with HRP-conjugated secondary antibodies (Jackson Immuno Research Laboratories, PA, USA) for 1 h at room temperature. The detection was carried out by using ECL chemilluniscence (Amersham Pharmacia, Buckinghamshire, United Kingdom). The antibodies used for this study are listed as follows: goat anti-beta-actin (1/5000; SC1616; Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-TH (1/1000; Millipore MAB 318), mouse anti-MAP-2 (1/1000; Millipore 05-346); rabbit anti- pAKT (1/2000; Cell signaling #9271) and rabbit anti-p PKC (1/2000; Cell signaling #9371).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons procedure as a post hoc analysis. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

K1-5 increased both neurite outgrowth and neurite connection in neuronal/glial cultures. As shown by β III tubulin staining, treatment with K1-5 resulted in a significant increase of neurite outgrowth and neurite connection in both cortical and mesencephalic cultures compared to non-treated control groups ((Figure 1A), 1B; $P < 0.05$). To our knowledge, this is the first time that K1-5 has been shown to enhance neurite outgrowth in the CNS. This finding was similar to its intact parental molecule, plasminogen, which has been shown to enhance neurite outgrowth from neocortical explant of rat brain and neuritogenesis [15,16]. Furthermore, Gutierrez-Fernandez et al demonstrated that the lysine binding site within K5 domain is required for the enhancement of neurite outgrowth [15]. Although kringle 1, 2 and 4 also have lysine binding sites, none of these kringles are required for neurite outgrowth. Therefore, it is reasonable to speculate the effect of K1-5 on neurite outgrowth may act through the interaction of receptors localizing on neuron surface and lysine binding sites within K5 domain.

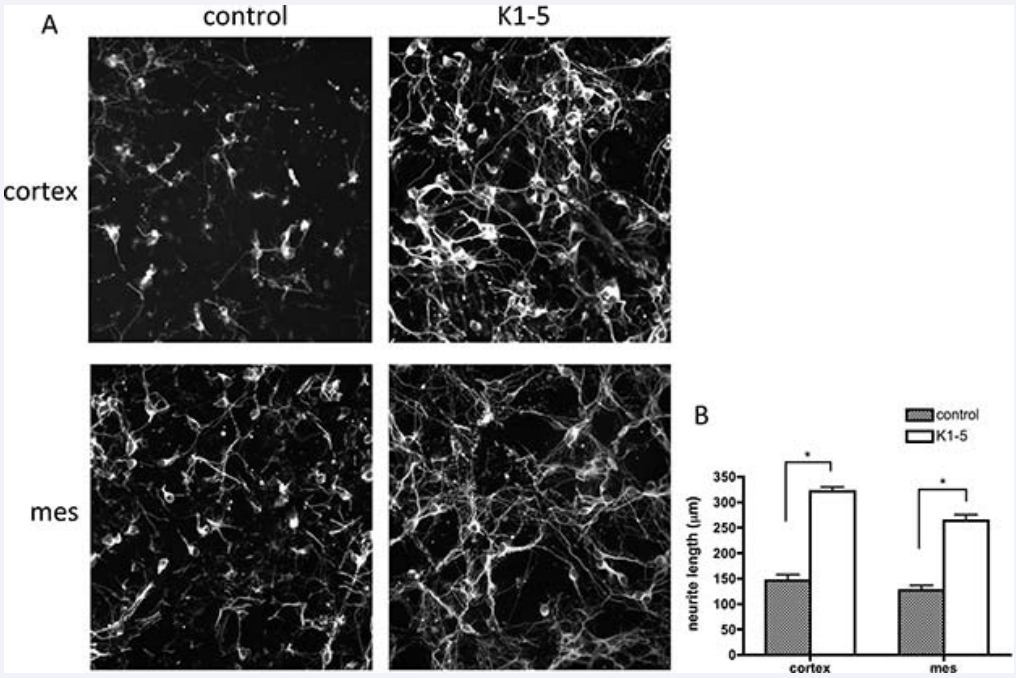

Figure 1 Kringle 1-5 (K1-5) enhanced neurite outgrowth in mixed neuronal/glia cell culture. (A) β III tubulin positive signals. (B) Quantification of neurite length. * P < 0.05.

K1-5 enhanced general neuron survival including dopaminergic neurons

Morphologically, β III tubulin positive neurons and dopaminergic neurons showed longer processes in the K1-5 treated mesencephalic cultures (Figure 2A,2B). Furthermore, the number of TH-positive dopaminergic neurons were proximally 2.5 fold increased in the K1-5 group compared to the control group (Figure 2C). Interestingly, treatment of mesencephalic cultures with MPP+, the percentage of cytosolic LDH release in medium was significantly reduced in the K1-5 treatment groups (Figure 2D). These results implied that K1-5 enhanced the survival of dopaminergic neurons. The number of surviving dopaminergic neuron was two-fold more in the presence of K1-5 than without K1-5 in the mesencephalic cultures. Here, there is another link of plasminogen acting on neurons as the number of dopaminergic neurons was significantly increased in the presence of plasminogen [17]. This study also demonstrated that plasminogen can increase dopamine uptake and the number of tyrosine-hydroxylase-expressing neurons in cultured rat mesencephalic neurons. In addition to its ability of protecting dopaminergic neurons, K1-5 is also involved in axonal sprouting of dopaminergic neurons.

K1-5 activated Akt and PKC pathway

Western blot analysis of mesencephalic cultures demonstrated that the expression level of MAP2 and TH were significantly increased in K1-5 group. Concurrently, K1-5 activated phosphorylation of both Akt and PKC in mesencephalic cultures (Figure 3). Although the precise cellular mechanism for K1-5 on dopaminergic neurons remains to be determined, we found that this effect was, at least in part mediated through Akt and PKC phosphorylation. In neurons, Akt pathway is classically described as a survival pathway in response to trophic factors such as nerve growth factor [18] and glial cell line-derived neurophic factor [19]. Akt signaling promotes cell survival through the inhibition of apoptosis [20]. The activation of Akt not only suppresses apoptosis [21,22] but also regulates axon growth and sprouting *in vitro* [23]. *In vivo*, it is also capable of inducing sprouting of adult mesencephalic dopamine neurons [24]. PKC has been implicated to function as a main neuronal survival pathway. Activation of PKC is part of the neuroprotective mechanisms in neuronal cells [25]. We demonstrated in the present study that the phosphorylation of Akt and PKC in the mesencephalic culture in the presence of K1-5. Taken together, it is suggested that similar to trophic factors, K1-5 provides neuroprotection by increasing both dopaminergic neuron survival and axonal sprouting through an activation of the Akt and PKC signal transduction pathways.

CONCLUSION

Our works, using neuronal/glial cultures from embryonic rat cortex and mesencephalic regions, have demonstrated that K1-5 increases neurite connection in cortex and mesencephalic regions as evidence by β III tubulin staining and MAP-2 expression. Especially in the mesencephalic culture, K1-5 increases the number of dopaminergic neurons (TH positive cells) and stimulates its neurite outgrowth. This effect was, at least in part mediated through Akt and PKC phosphorylation. In contrast, K1-5 had no effect on the number of GFAP and ED1 positive cells [5]. Furthermore, K1-5 protected the DA neurotoxin MPP^+-induced LDH release in these cultures. These results suggested that K1-5 acted directly on neurons and exerted beneficial/neuroprotective effects on neurons.

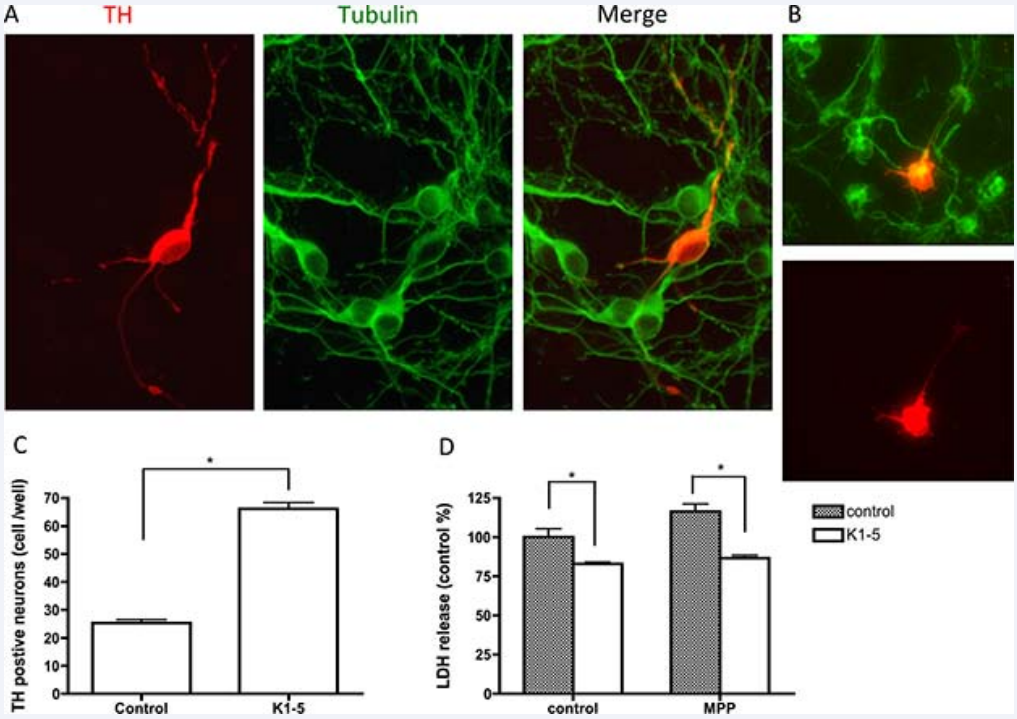

Figure 2 Analysis for effects of K1-5 on the survival of dopaminergic neurons. Cell survival was analyzed using immunostaining with anti-TH, a marker for dopaminergic neurons. (A) K1-5 group. (B) Control group. (C) Quantification of TH positive cells.(D) Released LDH in culture medium after treatment (K1-5 150ng/ml; MPP+ 10μM). * P < 0.05.

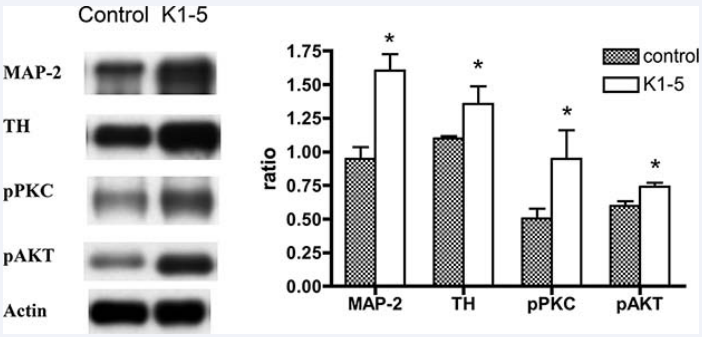

Figure 3 Effect of K1-5 on mesencephalic culture. Representative Western blots of the culture and quantification of protein levels. Significant differences were observed between control and K1-5 group. * P < 0.05.

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