—Communications—

Growth-stimulating Effect of Kallikrein on Rat Neural Stem Cells —II. Immunocytochemical Analysis and Specificity of the Enzyme for Neural Stem Cells—

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In a previous paper we reported a new function of tissue kallikrein: rat urinary kallikrein (RUK) has a marked growth-stimulatory effect on neural stem cells prepared from brains of prenatal rats. We here report that conspicuous differentiation of neural stem cells to neurons and/or glial cells did not occur during stimulation by kallikrein, and this growth-stimulating effect of kallikrein is considerably specific for neural stem cells, *i.e.*, RUK showed no detectable stimulatory effect on rat glial, PC12, GH_3, and HeLa cells. Thus this new function of kallikrein suggests the potential involvement of this enzyme on brain physiology. In addition, this effect of kallikrein for neural stem cells may have value in the treatment of cerebral ischemic stroke-induced injuries *etc.*, *i.e.*, kallikrein administered into the body stimulates neural stem cell proliferation and new neurons may be generated from them.

Key words——kallikrein; brain; neural stem cell; neuron; proliferation; cerebrum

INTRODUCTION

In general, tissue kallikrein (EC 3.4.21.36) is well known as a serine protease that has an extremely high substrate specificity for kininogen, which mainly exists in the plasma, and liberates a physiologically active peptide, kinin, and is widely distributed in the body, including the brain, of many species.[1—3] Kinin shows a variety of biological actions mainly through the kinin B_2-receptor.[4] In a previous paper, we reported a new function of tissue kallikrein, *i.e.*, rat urinary kallikrein (RUK), has a marked growth–stimulatory effect on neural stem cells prepared from the brains of prenatal rats without involvement of kinin release.[5] In the present paper, we investigated in more detail whether the differentiation of neural stem cells to neurons and/or glial cells occurs during this growth stimulation by kallikrein. Furthermore, specificities of the growth-stimulation of kallikrein for the neural stem cells were investigated.

MATERIALS AND METHODS

Animals Pregnant Wistar rats were obtained from Kyudou, Ltd., Fukuoka, Japan and fed ad libitum.

Rat Urinary Kallikrein (RUK) RUK preparations purified from the urine of Wistar rats according to our previously reported methods[6] were used. Purity of the enzyme was confirmed by SDS-polyacrylamide gel electrophoresis and Western blot analysis and a single band was observed.[3,6] The amounts of RUK added into the culture medium were calculated from the specific activity of RUK, 22.1 AU/mg.[6] One AU is the amount of enzyme that can hydrolyze 1 μmol prolyl-L-phenylalanyl-L-arginine-4-methyl-coumaryl-7-amide (Peptide Institute, Inc., Japan) per min at 30℃ and pH 8.0.

Neural Stem Cell Preparation Rat neural stem cells were prepared by the method of Tang et al.[7] with some modification.[5] In brief, embryonic striatal cells were obtained from timed pregnant Wistar rats at 17 days and the cells were cultivated on plastic dishes in 5 ml of DMEM/F12 containing N2 supplement (Invitrogen Co., USA), 20 ng/ml basic fibroblast growth factor (bFGF) (PeproTech EC, Ltd., UK), and 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich Co., USA) (culture medium I) per brain. After 3–5 days of culture, the cells formed floating neurospheres.

Growth-stimulatory Effect of RUK on Rat Neural Stem Cells and Immunohistochemistry Resuspension of the dissociated cells from neurosphere in culture medium I obtained by the same procedures as described in our previous paper[5] was seeded on a poly-L-lysine and laminin-coated[8] 96-well plate at a density of 8×10^3 cells/well with 100 μl of culture

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medium I. After 24-hour culture, RUK was added into the culture medium at the final concentration of 100 ng/ml and cell culture was continued. Culture media containing the same final concentration of RUK were changed every 3 days. The amounts of cells were assayed by Cell Counting Kit-8 (Wako Pure Chemical Industries, Ltd., Japan). For immunostaining, resuspension of the dissociated cells from neurosphere in culture medium I was obtained and the cells were seeded on poly-L-lysine and laminin-coated cover glasses at a density of 2.2×10^4 cells/cm^2. After 7 and 11 day's cultivation after the first addition of RUK in the culture medium, the cover glasses were taken out from the dishes and fixation with a Bouin's solution was carried out. Then, immunocytochemical staining was carried out by standard procedures using 0.5 μg/ml of anti-βIII tubulin mouse IgG (Promega Co., USA), 0.5 μg/ml of anti-nestin mouse IgG (Chemicon International Inc., USA), and anti-glial fibrillary acidic protein (GFAP) rabbit IgG (Dako, Denmark) for immunostaining as a primary antibody, and 1 μg/ml of Alexa Fluor 488 goat-rabbit IgG (Invitrogen Co.) and 1 μg/ml of Alexa Fluor 594 goat anti-mouse IgG (Invitrogen Co.) as a secondary antibody.

Effect of RUK on GH_3, PC12, and HeLa Cells GH_3, PC12, and HeLa cells were purchased from Dainippon Sumitomo Pharma Co., Ltd., Japan and cultivated on plastic dishes using Ham's F-10 (Invitrogen Co.) containing 2.5% (v/v) fetal bovine serum (FBS) (Cansera International Inc., Canada) and 15% horse serum (Invitrogen Co.) for GH_3 cells, RPMI1640 (Sigma-Aldrich, Inc.) containing 5% FBS and 10% horse serum for PC12 cells, and minimum essential media (MEM) (Invitrogen Co.) containing 10% FBS for HeLa cells. GH_3, PC12, and HeLa cells were seeded at a density of 2.6×10^4, 1.3×10^4 and 3.1×10^3 cells/cm^2, respectively. The effect of RUK on these cells was investigated using the same method as that of the neural stem cells as described above.

Effect of RUK on Rat Glial Cells Prepared from Neural Stem Cells A greater part of neural stem cells differentiated into glial cells when the cells were cultured in the presence of 10% FBS. Therefore the neural stem cells were cultured for 7 days in MEM containing 10% FBS to obtain GFAP-positive cell-rich preparation. After dissociation, the glial cell-rich cells were seeded with culture medium I at a density of 5×10^4 cells/cm^2 and the effect of RUK on these cells was investigated using the same method as described above.

RESULTS

Growth-stimulating Effect of RUK on Rat Neural Stem Cells and Immunocytochemical Analysis As shown in our previous paper (Fig. 2 and Table I in Ref. 5), a marked growth-stimulatory effect of RUK was observed in a dose-dependent manner at the final concentration of RUK 10—100 ng/ml. Thus immunocytochemical analysis of the cells at 7 and 11 days after the first addition of RUK (100 ng/ml) in the culture medium was carried out. The neural stem cell preparations obtained from the prenatal rat brain and used in the present investigation were nestin positive (Fig. 1(A)) and GFAP and βIII tubulin negative (Fig. 1(B)), indicating that the contamination of glial cells and neurons in the neural stem cell preparations was very little. Figures 1(C), (D), (E) and (F) are the results at 7 and 11 days after the first addition of RUK in the culture medium. As shown in Fig. 1 (C) and (E), most of the cells were nestin positive (97 and 96% of the total cells, respectively), and GFAP and βIII tubulin-positive cells were sparse even at 11 days after the first addition of RUK (Figs. 1(D) and (F), although a tendency for a slight increase of the GFAP-positive cells was observed with the lapse of time after the addition of RUK, suggesting that conspicuous differentiation of the neural stem cells to neurons and/or glial cells did not occur by RUK. The neural stem cells stimulated by RUK for 7 days maintained differentiating ability to glial cells and neurons (Fig. 1(G)). The neural stem cells also maintained differentiating ability to glial cells and neurons during stimulation of RUK (Fig. 1(H)). These observations support the result that RUK has a stimulatory-effect on neural stem cells without differentiation to glial cells and neurons.

Effect of RUK on GH_3, PC12, and HeLa Cells RUK showed no detectable growth-stimulating effect on PC12, GH_3, and HeLa cells at final RUK concentrations of 10 and 100 ng/ml (Fig. 2).

Effect of RUK on Rat Glial Cells The photo in Fig. 3 shows the glial cell-rich preparation prepared from rat neural stem cells and 95% and 3% of the total cells were GFAP- and βIII tubulin-positive, respectively. Using this cell preparation, the effect of RUK was investigated. As shown in the figure, no de-

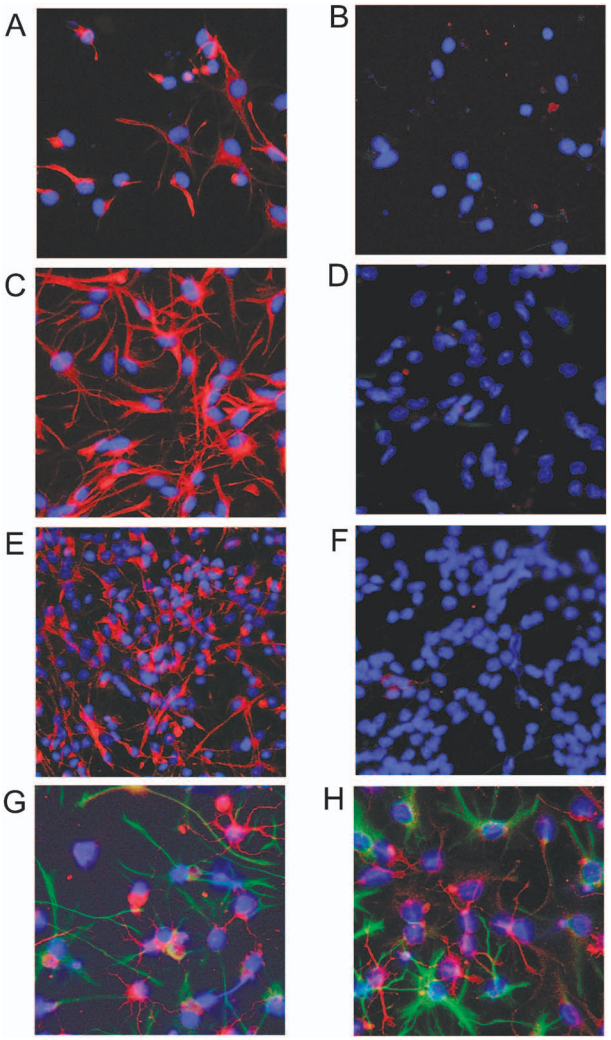

Fig. 1. Immunocytochemical Analysis of Rat Neural Stem Cell Preparations before and after Addition of RUK to Culture Medium

A, C, and E: Immunocytochemisty for nestin (red), B, D, and F: Immunocytochemistry for GFAP (green) and βIII tubulin (red), namely the mixture of antibodies for GFAP and βIII tubulin as a primary antibody and the mixture of Alexa Fluor 488 and 594 labeled antibodies for rabbit and mouse IgG as secondary antibody were used in these preparations. A and B: Before addition of RUK in culture medium, C and D: 7 days after first addition of RUK, E and F: 11 days after addition of RUK, G: Neural stem cells 7 days after first addition of RUK were further cultivated in RUK, bFGF, and EGF-removed culture medium I for 7 days, then immunocytochemical staining for GFAP (green) and βIII tubulin (red) was carried out. H: Neural stem cells 7 days after first addition of RUK were further cultivated in bFGF and EGF-removed culture medium I for 7 days under the presence of RUK, then immunocytochemical staining for GFAP (green) and βIII tubulin (red) was carried out. Nuclei were stained with DAPI (blue).

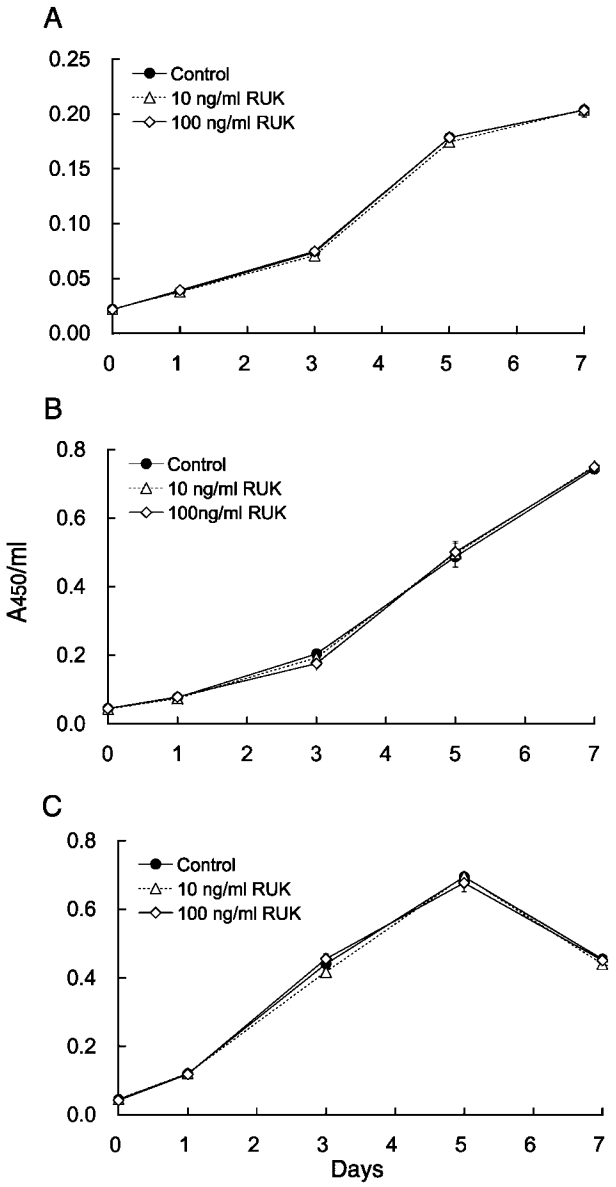

Fig. 2. Effect of RUK on Proliferation of GH_3, PC12, and HeLa Cells

A, B, and C: GH_3, PC12, and HeLa cells. Each point and the vertical bars are mean and S.E., respectively ($n=8$ for GH_3 and HeLa cells, $n=3$ for PC12 cells).

tectable effect was observed on this glial cell-rich preparation.

DISCUSSION

A novel function of tissue kallikrein, a growth-stimulatory effect of RUK on rat neural stem cells, was determined in our previous study.[5] The present work showed that conspicuous differentiation of neural stem cells to neurons and/or glial cells did not occur during stimulation by RUK and the differentiation ability of the neural stem cells was maintained after stimulation of RUK. Furthermore, RUK showed no significant stimulatory effect on the proliferation of rat glial cells (accurately, glial cell-rich preparation) prepared from the neural stem cells. RUK also showed no significant effect on PC12 cells, which are widely used in neuronal differentiation research. In the pituitary gland, kallikrein is specifically expressed

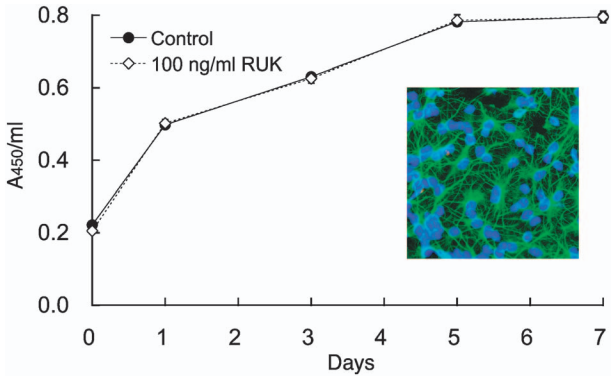

Fig. 3. Effect of RUK on Proliferation of Rat Glial Cells

Each point and the vertical bars are mean and S.E., respectively ($n=8$). The photo in the figure is the result of immunocytochemical staining for GFAP (green) and βIII tubulin (red) of the glial cell-rich preparation obtained from rat neural stem cells by the method described in Materials and Methods.

in prolactin-producing cells.[6] GH_3 cells are pituitary gland-derived cells and produce both kallikrein and prolactin. RUK also showed no significant effect on these cells. RUK showed no effect on HeLa cells. These observations suggest that the growth-stimulating effect of RUK is considerably specific for neural stem cells. Recently, using a gene-delivery approach or kallikrein protein infusion in rats, Chao's group reported a novel function of tissue kallikrein in the brain. The enzyme has a significant protective effect against cerebral ischemic stroke-induced injuries and tissue kallikrein may have value in the treatment of acute ischemic stroke.[9,10] The protective effect of kallikrein is mediated by kinin B_2-receptor activation via inhibition of apoptosis and inflammation and promotion of angiogenesis and neurogenesis, and kinin stimulates neuronal cell growth in the ischemic brain.[10] The results obtained in the present study suggest another mechanism for the protective effect of tissue kallikrein, *i.e*, kallikrein stimulates neural stem cell proliferation and new neurons may be generated from them. Other recent work has also revealed newly discovered functions of serine proteases, tissue plasminogen activator, in the central nervous system, *i.e.*, this fibrin clot degradation-associated enzyme has been shown to have roles in learning and memory, stress, neuronal degeneration, addiction, Alzheimer's diseases, *etc.*[11] These and the results obtained here strongly suggest the potential involvement of kallikrein in brain physiology, in addition to the liberation of kinin, although at present the real substrate(s) of the enzyme involved in this function is unknown.

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