Volume 314, number 2, 167–170 FEBS 11856 December 1992

Retinoic acid enhances the secretion of plasminogen from cultured rat microglia

Kazuyuki Nakajima, Nagisa Takemoto and Shinichi Kohsaka

Department of Neurochemistry, National Institute of Neuroscience, Kodaira, Tokyo 187, Japan

Received 14 September 1992

To determine the amount of plasminogen in microglial conditioned medium, a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) for rat plasminogen was established. Weak cross-reactivity with human serum plasminogen was observed, while no reactivity was detected with frog and carp plasminogen. The specificity of the immunosorbent assay was confirmed by Western blotting. The secretion of plasminogen into the microglial culture medium was quantified by using the established ELISA and was found to be increased depending on the culture time and number of microglia. The secretion was increased about 5-fold by stimulation with retinoic acid, while interleukin-1, and basic fibroblast growth factor showed no significant effect.

Microglia; Plasminogen, Enzyme-liked immunosorbent assay, Culture; Conditioned medium

1. INTRODUCTION

Microglia have been thought to be implicated not only in regeneration after injury or pathological damage but also in morphogenesis during development of the central nervous system (CNS) [1]. To study these events, methods for isolation of microglia and an in vitro culture system have been developed [2–5]. So far, isolated and cultured microglia have been reported to produce the biologically active substances: nerve growth factor (NGF) [6], interleukin-1 (IL-1) [7], IL-6 [8], tumor necrosis factor (TNF) [9,10] and basic fibroblast growth factor (bFGF) [11]. Furthermore, we recently found that microglia secrete some serine-type proteases, which were identified as elastase [12] and a urokinase-type plasminogen activator (uPA) [13]. More recently, plasminogen, which is a specific substrate of PA, was also detected in microglial conditioned medium (Mic-CM) by zymography and Western blotting [14]. The de novo synthesis of plasminogen was also demonstrated in microglia [14]. However, how plasminogen secretion from microglia is regulated remained unknown. To obtain further information about microglia-derived plasminogen, we investigated the effect of retinoic acid, which is known to accelerate microglial differentiation [2], on the secretion of plasminogen from microglia. The effect of bFGF and IL-1, as stimulators of PA secretion by microglia [13], was also investigated.

Correspondence address: S. Kohsaka, Department of Neurochemistry, National Institute of Neuroscience, Kodaira, Tokyo 187, Japan. Fax: (81) (423) 46-1751.

2. MATERIALS AND METHODS

2.1. *Preparation of microglial conditioned medium*

The isolation of rat microglia from a primary culture of Day 1 rat brain and the preparation of microglial conditioned medium (Mic-CM) were described previously [5,12,13]. Microglial cells were allowed to adhere to 6- or 12-well plates (Costar). After removal of non-attached cells, the microglia were washed 3 times with serum-free Dulbecco's modified Eagle medium (DMEM), and incubated with 0.5 ml (for 12-well plates) or 1 ml (for 6-well plates) of the same medium in the presence or absence of retinoic acid, IL-1 (Genzyme) and bFGF (R&D Systems). The recovered conditioned medium was centrifuged at 1,500 rpm for 10 min and the supernatant was used for ELISA either immediately or after storage at −80°C.

2.2. *Purification of plasminogen and preparation of the antibody*

Plasminogen was purified from rat (Wistar), frog (*Rana catesbeiana*) and carp (*Cyprinus*) plasma by lysine-Sepharose [15] and Sephadex G-150 column chromatography. Human plasminogen was provided by Sigma. Antiserum against rat plasminogen was raised in our laboratory by injection of highly purified rat plasminogen into rabbits as described previously [14], and the IgG was prepared by using an Immuno Pure IgG Purification Kit (Pierce).

2.3. *Establishment of ELISA for rat plasminogen*

ELISA for rat plasminogen was developed to detect and quantify plasminogen secreted from microglia into the culture medium. Rat, human, carp and frog plasminogen dissolved in 200 μl of DMEM, or 200 μl of Mic-CM was adsorbed to microwells of 96-well microtiter plates (Costar) for 24 h at 4°C. The wells were washed twice with PBS containing 0.05% Tween-20 (PBS-Tw), PBS containing 0.5% BSA was added and the plates were incubated for 30 min at room temperature. PBS containing 1% normal goat serum (PBS-NGS) was transferred to the wells, anti-rat plasminogen antibody (3.9 μg of IgG/ml) was added and the plates were incubated overnight at 4°C. The wells were washed four times with PBS-Tw, peroxidase-conjugated goat anti-rabbit IgG (1:500) in PBS-NGS was added and the plates were incubated for 30 min. After the wells were washed six times with PBS-Tw, a solution of 1.0 mg of *o*-phenylenediamine per ml in 0.04% H_2O_2 was added, the plates were incubated again and the absorbance was measured at 490 nm in a 96-well microplate assay spectrophotometer (NJ 2000).

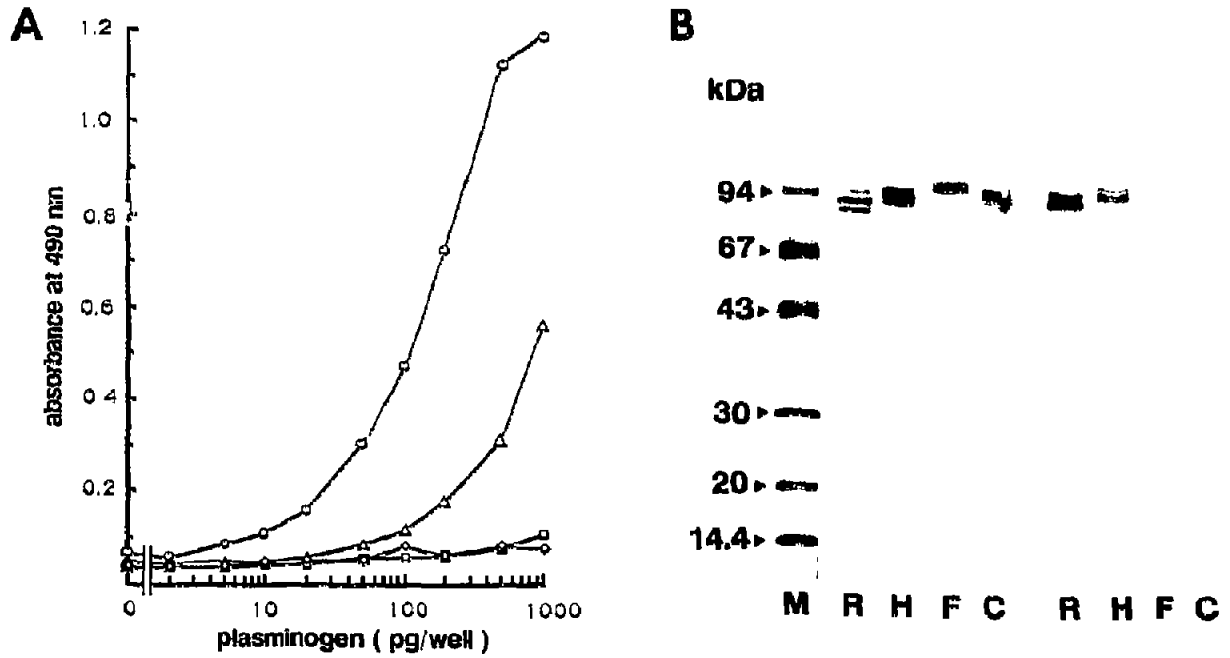

Fig. 1. Standard assay curve for rat plasminogen and cross-reactivity with plasminogens of other species. (A) Present ELISA. The indicated amounts of rat plasminogen (○), human plasminogen (△), frog plasminogen (□) and carp plasminogen (◇) were measured by ELISA. The value is expressed as the mean of duplicate assays. (B) SDS-PAGE and Western blotting analysis. One μg quantities of rat plasminogen (lane R), human plasminogen (lane H), frog plasminogen (lane F) and carp plasminogen (lane C) were subjected to SDS-PAGE under the nonreducing condition, electroblotted to Immobilon and stained with Coomassie brilliant blue (left side). Lane M shows molecular weight marker proteins. Five ng of each plasminogen was also subjected to SDS-PAGE and electroblotted. The Immobilon membrane was immunostained with anti-rat plasminogen antibodies (right). The order is the same as that in the left panel.

2.4. Immunoblotting analysis

Plasminogen or Mic-CM was subjected to SDS-PAGE under the non-reducing condition and electroblotted to Immunobilon-P (Millipore) as described previously [14]. The Immobilon was incubated with rabbit antibodies against rat plasminogen (20 μg of IgG/ml) for 1 h. After being washed, the membrane was incubated with peroxidase-goat anti-rabbit IgG (×200). The antigen–antibody complexes were stained with diaminobenzidine and H_2O_2.

3. RESULTS AND DISCUSSION

3.1. Establishment of ELISA for rat plasminogen

As reported previously [14], we could detect plasminogen in concentrated or freeze-dried Mic-CM by UK-zymography or Western blotting. In those studies, however, the exact amount of plasminogen could not be determined. To quantify the small amount of plasminogen, a highly sensitive and specific ELISA was established. A standard curve of rat plasminogen and the cross-reactivity of the antibody with plasminogen from other species are shown in Fig. 1A. The detection limit was approximately 20 pg per well. The assay was specific for rat plasminogen, showing weak cross-reactivity with human plasminogen (about 15%) and no reactivity with frog and carp plasminogen. The immuno-specificity of anti-rat plasminogen antibody against four kinds of plasminogen was also examined by Western blotting (Fig. 1B). As expected, anti-rat plasminogen antibody reacted strongly with rat plasminogen and relatively weakly with human plasminogen, but not with frog and carp plasminogen.

3.2. Determination of plasminogen in Mic-CM

The amount of plasminogen in the Mic-CM was measured by ELISA. The amount of secreted plasminogen increased depending on the number of cultured cells (Fig. 2A) and culture time (Fig. 2B). The assay was highly sensitive; the detection limit was 20 pg/well (100 pg/ml), which was sufficient for measurement of plasminogen in the Mic-CM. In earlier studies, the limits of detection of plasminogen were 5 ng by zymography, 1 ng by chromogenic assay and 0.2 ng by Western blotting. Bohmfalk and Fuller [16] reported an assay method for rat plasminogen. However, the range of linearity in the assay was 6–90 ng/ml, indicating that the present assay is more sensitive than their method.

3.3. Influence of effectors on the plasminogen secretion

Fig. 3 summarizes the influence of various effectors on the secretion of plasminogen. Lipopolysaccharide, which is the most potent stimulant of IL-1 [4], TNF [10], and NGF [6] secretion by microglia, increased the secretion of plasminogen as reported previously [14]. Retinoic acid, one of the differentiation agents [17] for microglia [2], markedly promoted the plasminogen secre-

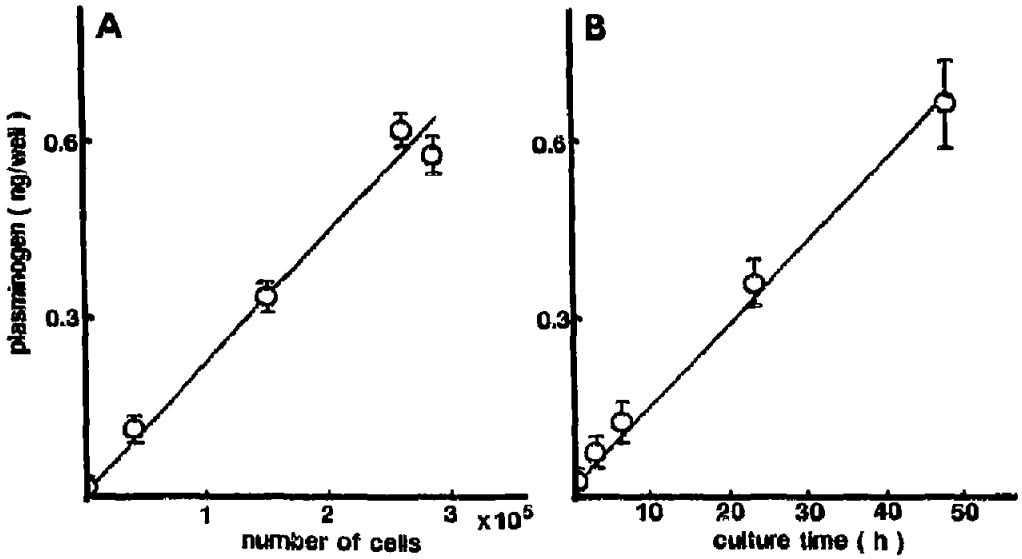

Fig. 2. Secretion of plasminogen from cultured microglia. (A) Cell number dependency. The indicated numbers of microglia were seeded into 6-well plates and cultured for 24 h as described in section 2. The recovered CM was directly assayed in duplicate. The value is expressed as the mean of duplicate assays. (B) Time course. Microglia (1.5×10^5 cells) were seeded into 6-well plates and cultured. At the indicated culture times, CM was recovered and directly assayed as in Fig. 2A.

tion by microglia. However, IL-1 and bFGF, which are strong stimulators of uPA secretion by microglia [13] showed no significant effect. As shown in Fig. 4A, retinoic acid increased plasminogen secretion in a dose-dependent manner. In this experiment, the cell number was not affected. These results were confirmed by Western blotting analysis (Fig. 4B). These results suggest that retinoic acid is a stimulator of plasminogen secretion.

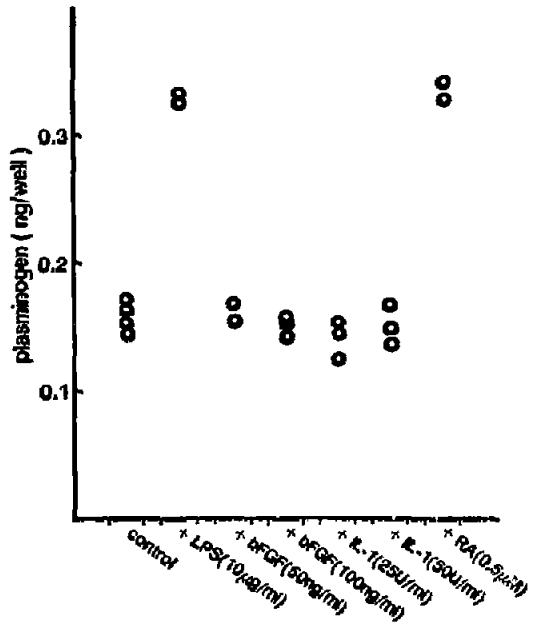

Fig. 3. Influence of effectors on the plasminogen secretion. Microglia (5×10^4 cells) were seeded into 12-well plates and cultured for 24 h in the presence of the indicated effectors. The recovered CM was directly assayed in duplicate to tetraplicate. LPS, lipopolysaccharide; RA, retinoic acid.

Giulian and Baker [2] found that the addition of retinoic acid changed the morphology of the ameboid type of microglia into the process-bearing type, which showed weak phagocytic, weak proliferating, weak non-specific esterase and weak acetylated LDL (low density lipoprotein)-binding activities. Those studies together with the present findings suggest that substances like retinoic acid may regulate the functional state of microglia which is closely associated with the changes in the secretion of plasminogen by microglia.

It is well known that the plasminogen–plasmin system has a potential ability for the activation, like proinsulin [18], proTGFβ [19], procollagenase [20], prouPA [21] and proIL-8 [22]. Also the plasminogen–plasmin system is involved in neurite extension [23–25], cell migration [26], and astroglial proliferation [27] in the CNS. In this sense, this protease zymogen may be involved in the control of neuronal function and/or neuronal growth. Further studies on the regulation of plasminogen secretion by microglia and the role of microglia-derived plasminogen in the brain are in progress. In addition, the assay described here is considered to be of great value in the detection of a small amount of plasminogen derived from various cells.

Acknowledgements. This study was supported by the Ministry of Health and Welfare, by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture and by the Japan Health Science Foundation.

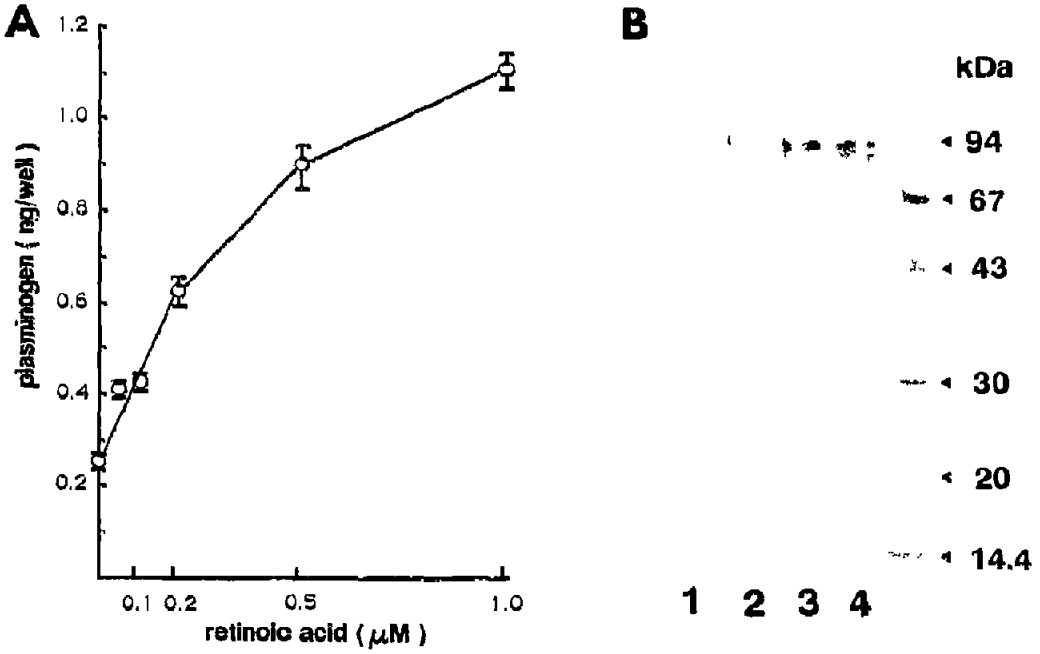

Fig. 4. Effect of retinoic acid on plasminogen secretion by microglia. (A) Determination by ELISA. Rat microglia (1.5×10^5 cells) were seeded into 6-well plates and further maintained in the presence of the indicated concentrations of retinoic acid. CM was collected 24 h after stimulation and directly assayed. The amount is expressed as the mean of duplicate measurements. (B) Western blotting analysis. Five hundred μl of control medium (lane 1) and medium stimulated with 0.2 μM (lane 2), 0.5 μM (lane 3) and 1.0 μM (lane 4) retinoic acid as in A were freeze-dried and Western blotted as in Fig. 1B.

REFERENCES

[1] Perry, V.H. and Gordon, S (1988) Trends Neurosci. 11, 273–277.
[2] Giulian, D. and Baker, T.J. (1986) J. Neurosci. 6, 2163–2178.
[3] Suzumura, A., Mezitis, S.G.E., Gonatas, N.K. and Silberberg, D.H. (1987) J. Neuroimmunol. 15, 263–278.
[4] Gebicke-Haerter, P.J., Bauer, J., Schobert, A. and Northoff, H. (1989) J. Neurosci. 9, 183–194.
[5] Nakajima, K., Hamanoue, M., Shimojo, M., Takei, N. and Kohsaka, S. (1989) Biomed. Res. 10 (S3), 411–423.
[6] Mallat, M., Houlgatte, R., Brachet, P. and Prochiantz, A. (1989) Dev. Biol. 133, 309–311.
[7] Giulian, D., Baker, T.J., Shin, L.-C.N. and Lachman, L.B. (1986) J. Exp. Med. 164, 594–604.
[8] Frei, K., Malipiero, U.V., Leist, T.P., Zinkernagel, R.N., Schwab, M.E. and Fontana, A (1989) Eur. J. Immunol 19, 689–694.
[9] Frei, K., Siepl, C., Groscurth, P., Bodmer, S., Schwerdel, C. and Fontana, A. (1987) Eur. J. Immunol. 17, 1271–1278.
[10] Sawada, M., Kondo, N., Suzumura, A and Marunouchi, T. (1989) Brain Res. 491, 394–397.
[11] Shimojo, M., Nakajima, K., Takei, N., Hamanoue, M and Kohsaka, S. (1991) Neurosci. Lett. 123, 229–231.
[12] Nakajima, K., Shimojo, M., Hamanoue, M., Ishiura, S., Sugita, H. and Kohsaka, S. (1992) J. Neurochem. 58, 1401–1408.
[13] Nakajima, K., Tsuzaki, N., Shimojo, M., Hamanoue, M. and Kohsaka, S. (1992) Brain Res. 577, 285–292.
[14] Nakajima, K., Tsuzaki, N., Nagata, K., Takemoto, N. and Kohsaka, S. (1992) FEBS Lett. (in press).
[15] Deutsch, D.G. and Mertz, E.T. (1970) Science 170, 1095–1096
[16] Bohmfalk, J.F. and Fuller, G.M. (1980) Science 209, 408–410.
[17] Wang, S.Y., Larosa, G.J. and Gudas, L.J. (1985) Dev. Biol. 107, 75–86.
[18] Virji, M.A.G., Vassalli, J.-D., Estensen, R.D. and Reich, E. (1980) Proc. Natl. Acad. Sci. USA 77, 875–879.
[19] Rifkin, D.B., Moscatelli, D., Bizik, J., Quarto, N., Blei, F., Dennis, P., Flaumenhaft, R. and Mignatti, P. (1990) Cell Differ. Dev. 32, 313–318.
[20] Liotta, L.A., Goldfarb, R.H., Brundage, R., Siegal, G.P., Terranova, V. and Garbisa, S. (1981) Cancer Res. 41, 4629–4636.
[21] Blasi, F., Vassalli, J.-D. and Dano, K. (1987) J. Cell Biol. 104, 801–804.
[22] Nakagawa, H., Hatakeyama, S., Ikesue, A. and Miyai, H. (1991) FEBS Lett. 282, 412–414.
[23] Kryostosek, A and Seeds, N.W. (1981) Science 213, 1532–1534.
[24] Pittman, R.N. (1985) Dev. Biol. 110, 91–101.
[25] Pittman, R.N., Ivins, J.K. and Buettner, H.M. (1989) J. Neurosci. 9, 4269–4286.
[26] Kalderon, N. (1979) Proc. Natl. Acad. Sci. USA 76, 5992–5996.
[27] Kalderon, N. (1982) J. Neurosci. Res. 8, 509–519.