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Regulation of cell migration and cytokine production by HGF-like protein (HLP) / macrophage stimulating protein (MSP) in primary microglia

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ABSTRACT

HGF-like protein (HLP)/macrophage stimulating protein (MSP) is the only structural relative of hepatocyte growth factor (HGF), and is involved in the regulation of peripheral macrophage activation. However, the actions of HLP in microglia, a species of macrophage in the nervous system, which is closely involved in the neural degeneration and regeneration, is not yet understood. This study found that Ron, the receptor for HLP, is expressed in primary microglia using RT-PCR, immunocytochemical staining and Western blotting, and, thus, sought to elucidate the function of HLP on the primary microglia. HLP promoted microglial migration without affecting cell survival and proliferation. Furthermore, real-time quantitative RT-PCR analysis revealed that HLP greatly increased the mRNA of inflammatory cytokines, including IL-6 and GM-CSF, and iNOS. These findings provide the first evidence that HLP has the potential to modulate inflammatory actions of microglia, which proposes novel aspects for the process of degeneration and/or regeneration of the brain.

HGF-like protein (HLP) was found as a molecule containing kringle domains and named for its high homology with hepatocyte growth factor (HGF) (10, 21, 22). Later, HLP was revealed to be identical to the macrophage stimulating protein (MSP) (15, 25). The actions of HLP are most commonly involved in the inhibitory process of macrophage activation (32). HLP inhibits lipopolysaccharide (LPS)- and cytokine-induced nitric oxide production in macrophage in vitro, and reduces the nuclear translocation of nuclear factor-κB (NF-κB). Three different null mutants of *Mst1r*, coding the HLP receptor/Ron were generated with different targeting strategies, and studies on these mutant mice showed that HLP functions to suppress the excess inflammatory responses of peripheral macrophages (3, 16, 30, 31).

Microglia is a kind of domestic macrophage, typically residing in the central nervous system (CNS). Microglia serve as scavenger cells by proliferation and migration towards the affected sites in response to neurodegenerative diseases, such as Alzheimer's, Parkinson's disease and amyotrophic lateral sclerosis (ALS), and elicit actions involved in regenerative and destructive processes (19). Typically, microglia releases neurotrophic cytokines, interleukin (IL)-1α and IL-6 (1), and the family of neurotrophins such as nerve growth factor (NGF), neurotrophin (NT)-3 and brain-derived neurotrophic factor (BDNF) (18). These studies have pointed out that the so-called activated microglia possess reciprocal functions, i.e. neuroprotection and neurodestruction.

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The expression of HLP mRNA in adult rodent brain tissue is revealed by Allen Brain Atlas on the web site (http://allenbrainatlas.com). This study focused on the function of HLP on microglia. Here we show that microglia is one of the target cells of HLP, and that the cell migration and production of inflammatory cytokines are enhanced by HLP-stimulation in primary microglia. These findings predict that the novel roles of HLP involved in the regulation of microglial activation, which leads to neuroprotection and neurodestruction in the brain.

MATERIALS AND METHODS

Recombinant human HLP was purified from conditioned media of COS-7 cells transfected with an expression vector containing human HLP cDNA as described previously (6, 25), and the purity of HLP was confirmed to be more than 95% by SDS-PAGE and silver-staining.

C57BL/6 mice were purchased from SLC (Shizuoka, Japan). All efforts were made to minimize the number and discomfort of animals.

We prepared murine primary microglia by the same methods of preparation of rat microglia with identical purity (> 95%) (8). Murine microglia were cultured in modified N3 (mN3) medium containing DF (high glucose DMEM/Ham's F12, 50 : 50) medium supplemented with 10 μg/mL insulin, 100 μg/mL apo-transferrin, 20 nM progesterone, 50 μM putrescine, and 30 nM sodium selenite (7). Murine primary cerebral cortex neurons and astrocytes were cultured as described (7). Murine macrophage cell line J774A.1 were cultured as previously described (20).

For reverse transcriptase (RT-) PCR and real-time quantitative RT-PCR, total RNA was isolated from microglia cultured for one day. One μg of total RNA was reverse-transcribed into first-strand cDNA with a random hexaprimer using Superscript II reverse transcriptase (Lifescience Technologies Inc, Grand Island, NY). To detect *Ron*, RT-PCR analyses were performed using forward primer, 5'-AGG TTT TCC GTC GCT GTC-3', and reverse primer, 5'-GCC TGA AGC ACT GGG TAG-3'. Plasmid inserted murine *Ron* cDNA was used as a positive control. For the quantitative PCR analyses, the primers and probes for IL-1α, IL-1β, IL-6, tumor necrosis factor (TNF)α, granulocyte-macrophage colony-stimulating factor (GM-CSF), inducible nitric oxide synthase (iNOS), BDNF, ciliary neurotrophic factor (CNTF), HLP, Ron and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Foster City, CA). Sequences for primers and TaqMan fluorogenic probes of murine HGF were as follows: forward primer, 5'-AAG AGT GGC ATC AAG TGC CAG-3', reverse primer, 5'-CTG GAT TGC TTG TGA AAC ACC-3', probe, 5'(FAM)-TGA TCC CCC ATG AAC ACA GCT TTT TG- (TAMARA)3'. The PRISM 7000 real-time PCR system (Applied Biosystems) was used for the amplification and online detection. Experimental samples were matched to the standard curve generated by amplifying serially diluted products, using the same PCR protocol. GAPDH cDNA was also amplified to decide the amount of each template cDNA.

Immunocytochemical analyses were performed as follows. Microglia were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and reacted with rat anti-Mac1 antibody (a specific marker for microglia; 1 : 100, BD Biosciences, San Jose, CA) and rabbit anti-Ron antibody (1 : 100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 5% goat serum and 0.3% Triton X-100. Then cells were rinsed with PBS and labeled with Alexa488-conjugated goat anti-rabbit IgG (1 : 500) and Alexa546-conjugated goat anti-rat IgG (1 : 500). To confirm the specificity of anti-Ron antibody, the antibody was preincubated with immunized Ron peptides (Santa Cruz Biotechnology, Inc.). The fluorescence images were obtained using an LSM5 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Microglia and J774A.1 cells were lysed for Western blotting in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 25 mM β-glycerophosphate, 150 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 2.5 μg/mL antipain, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 5 μg/mL leupeptin. After removal of the debris, equal amounts of lysed-proteins were separated on SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and subjected to Western blotting using mouse anti-Ron monoclonal antibody (1 : 200, BD Biosciences). Signals were visualized using HRP-coupled secondary antibody and an ECL detection kit (GE Healthcare UK Ltd, Buckinghamshire, UK).

Cell survival and proliferation were assayed as follows. The cells were seeded onto 12-well plates at 5×10^4 cells/well, and cultured in both the presence and absence of HLP (10 ng/mL) for 24 and 48 h. The survival rate of microglia was scored by double staining with Calcein-AM (Wako Pure Chemical Industries, Osaka, Japan) and propidium iodide (PI). The fluorescence images were obtained using

an LSM5 laser scanning confocal microscope (Carl Zeiss). The viable cells were stained green with Calcein-AM and naked nuclei of the dead cells were stained red with PI. The number of viable or dead cells in three different fields in a well was enumerated and the resulting scores were determined from the scores in three wells. A proliferation index was measured from the number of cells. Results were expressed as mean ± S.E.

Migration of microglia was assayed using a transwell system with HTS Fluoro Blok inserts (pore size 8 μm) (BD Biosciences). Microglia (5×10^4 cells/insert) were placed onto the upper chamber pre-coated with fibronectin (Lifescience Technologies Inc.), and HLP was added to the medium in the bottom chamber (Fig. 3A). Cells were incubated under 5% CO_2 at 37°C for 24 h, and stained with Calcein-AM. Images of the bottom side of the membrane were obtained using an LSM5 (Carl Zeiss). Five different fields were chosen in each well and the number of migrated cells was enumerated.

An ANOVA test was used to compare several differently treated groups, and this test was followed by a post hoc test, which takes multiple testing into account using Statview software (SAS Institute, Cary, NC).

RESULTS

Expression of Ron in murine microglia

To determine which cell species express the Ron, HLP-receptor, in the CNS, RT-PCR analysis was performed using cDNA synthesized from the total RNA of primary cultured microglia, astrocytes and cerebral cortex neurons prepared from C57BL/6 mice (Fig. 1A). A specific primer set for Ron extracellular domain was designed to detect a full-length form of Ron, since Ron lacking the extracellular domain was found in hematopoietic cells (12). As the negative control templates in the PCR analysis, the reverse transcriptase-free cDNA synthesis reaction mixture {RT(-) in Fig. 1A right panel} and the template-free mixture {template (-) in Fig. 1A left panel} were tested, and no PCR product was amplified from them. On the other hand, the PCR products were evidently amplified from the cDNA of all tested neural cells, and the expression levels of the *Ron* mRNA were estimated to be almost equal between those cells (Fig. 1A, left panel). The PCR products in these studies coincided with the products from the *Ron* positive control vector in their size (*Ron* plasmid in Fig. 1A left panel).

In the immunocytochemical staining, the most of

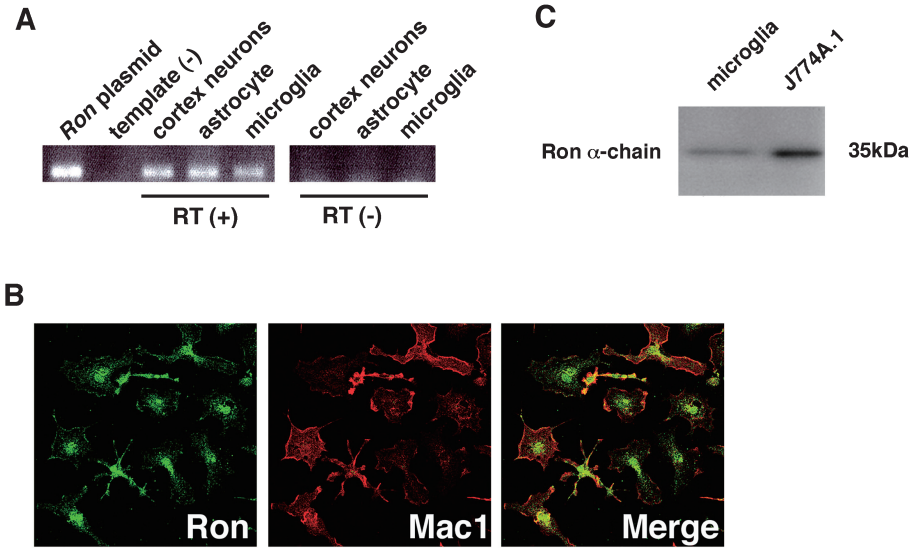

Fig. 1 Ron is expressed in microglia. (A) RT-PCR analysis. Microglia were cultured in mN3 medium for 24 h; astrocytes were cultured in DF medium containing 10% FBS for 2 weeks and contaminated cells were detached and removed by shaking culture dishes; and cortex neurons were cultured in mN3 medium for 2 days. *Ron* mRNA was detected in microglia, astrocytes, and cerebral cortex neurons; RT+, in the presence of reverse transcriptase in a reverse transcription step; RT–, in the absence of reverse transcriptase. A plasmid containing a *Ron* sequence served as a positive control. (B) Immunocytochemical analysis for Ron. Red signal shows anti-Mac1 immunoreactivity (IR) and green signal shows anti-Ron IR. Mac1 positive cells, indicating microglia, expressed Ron protein. (C) Western blotting for Ron in microglia and J774A.1 cells. A 35-kDa band, corresponding to the size of Ron α-chain, was detected in cultured microglia and the murine macrophage cell line J774A.1 using an antibody specific for Ron α-chain. J774A.1 cells served as a positive control for Ron.

cells in our microglial culture was Mac1 positive and the Ron staining signals were detected at the edge of cell shape in those cells, showing that Ron is expressed on the cytoplasmic membranes of microglia (Fig. 1B). Additionally, the Ron signals were closely located in cell nuclei (Fig. 1B), indicating the same staining pattern as the subcellular localization of HGF-receptor/c-Met tyrosine kinase, the structural relative of Ron (27). These signals in immunocytochemical staining for Ron could be eliminated using anti-Ron antibody preincubated with its blocking peptide (data not shown). This also means that the PCR product from microglial culture in Fig. 1A reflect the expression of Ron in microglia but not in contaminating neural cells. The results indicate that microglia is one of the Ron-expressing cell species.

Functional Ron consists of an α- and a β-chain, which are processed from a single proform polypeptide and the alternatively deleted Ron mRNA codes nonfunctional Ron which lacks extracellular domain (12, 31). Therefore, Western blotting was performed using an antibody specific for the α-chain, present in the extracellular domain, to evaluate the expression of functional Ron. Cell lysates of the primary microglia and, as the positive control, murine macrophage cell line J774A.1 were prepared and subjected to Western blotting. A 35-kDa band, corresponding to the size of the Ron α-chain, was clearly detected in both primary microglia and J774A.1 cells (Fig. 1C).

These data revealed that the functional (processed) form of full-length Ron is expressed in microglia.

Effect of HLP on microglial migration

The accumulation of microglia at the affected and marginal regions in the degenerated and injured brain is observed as the results of stimulated proliferation and/or migration of the cells, which are typical actions of the activated microglia (19). At first, the possible effect of HLP on microglial migration was assessed using a transwell chamber system (Fig. 2A). rhHLP (3, 10 ng/mL) was added in the bottom compartment of the chamber and the cells were seeded in the upper compartment which was separated from the bottom chamber with a micropored membrane. After 24 h incubation, the number of cells on the bottom side of the membrane was counted (Fig. 2B and C). Compared with the results from unstimulated wells, a 2- to 3-fold number of cells was detected when HLP was added (Fig. 2C). These results showed that HLP enhances microglial migration and/or proliferation.

Less effect of HLP on microglial survival and proliferation

Since it is reported that HLP promotes keratinocyte survival (4) and proliferation (32), the possibility of these effects of HLP on microglia were assessed. The primary microglia were cultured in the absence and the presence of 10 ng/mL HLP for up to 48 h, and the population of viable and dead cells was analyzed by double staining with Calcein-AM and PI, respectively. With this method, the viable cells exhibited a green signal and dead cells exhibited a red signal (Fig. 3A). The survival ratio at 24 h after seeding was more than 80% in the absence of HLP, and it decreased to 50% 48 h after seeding. This survival profile was nearly equivalent even in the presence of HLP (Fig. 3A, B). The total number of microglia at 48 h after seeding was unchanged in either the presence or in the absence of HLP, indicating that HLP failed to affect the microglial proliferation. Together, the difference in the cell number detected between HLP-presence and -absence in migration assays (Fig. 2B and C) shows that HLP enhanced the cell migration, but not proliferation, in microglia.

Effect of HLP on regulation of cytokine mRNA in microglia

Another feature of the activated microglia is the production of many kinds of cytokines and neurotrophic factors. These mediate either the degenerative or the regenerative functions of microglia in the CNS. To investigate whether HLP changes the expression level of cytokines and neurotrophic factors in microglia, the level of their mRNA was measured (Table 1). Microglia were cultured in the presence and absence of 10 ng/mL HLP for 24 h, and their total RNAs were isolated and subjected to quantitative real-time RT-PCR analysis. The results showed that HLP-treatment decreased HGF and HLP mRNA levels, whereas the treatment did not alter the levels of other neurotrophic factors such as BDNF and CNTF. Similarly, the *Ron* mRNA level did not change with HLP-treatment. On the other hand, the mRNA of proinflammatory cytokines IL-1α, IL-1β, IL-6, TNFα and GM-CSF were markedly upregulated in the presence of HLP. Compared with their controls (HLP-untreated), IL-6 and GM-CSF increased more than 200-fold, and TNFα achieved more than 2.5-fold under the HLP-treatment.

iNOS was upregulated almost 150-fold by HLP-treatment in microglia (Table 1), It is noteworthy that iNOS was suppressed 0.7-fold by HLP in the mouse macrophage cell line, J774A.1(data not

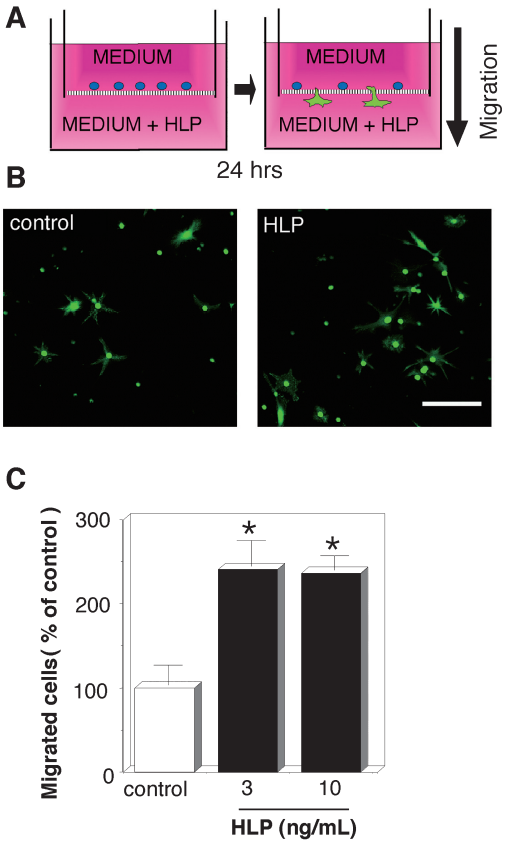

Fig. 2 HLP promotes microglial migration. (A) Schematic representation of the transwell chamber used for migration assays of cultured microglia. Scale bars, 100 µm. (B) Calcein-AM staining of migrated microglia. Microglia translocated to the bottom side of the transwell chamber membrane was stained with Calcein-AM (Green). Left panel shows the effects of the culture for 24 h in the absence of HLP, and right panel shows the effects for 24 h in the presence of 10 ng/mL HLP. (C) Quantification of microglia migrated to the bottom side of the membrane. The number of cells in the absence of HLP was defined as 100%. In HLP-treated wells (3, 10 ng/mL), 2- to 3-fold increase of migrated microglia, compared with controls, were observed ($P < 0.05$). Experiments were done in triplicates and results are shown as mean ± S.E.

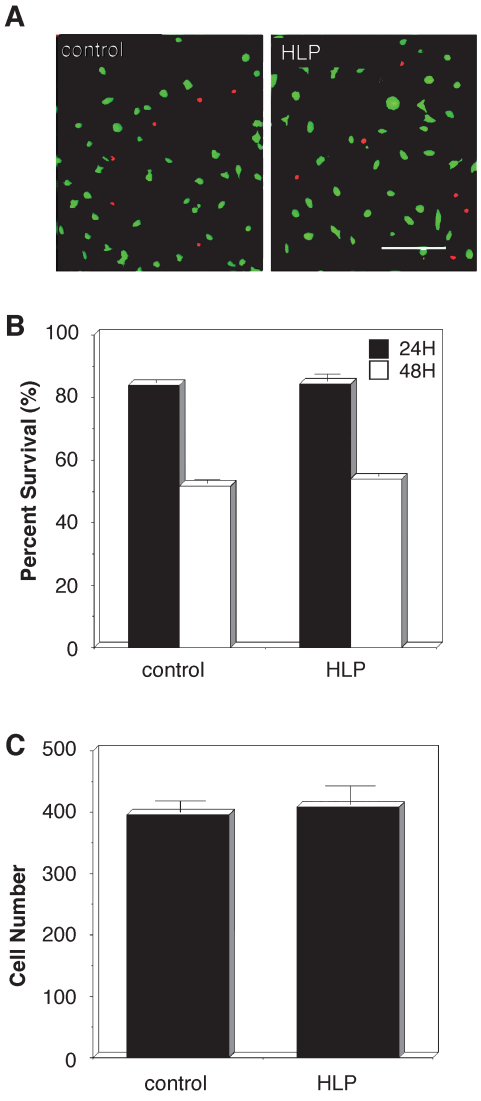

Fig. 3 HLP does not affect microglial survival and proliferation. (A) Double staining of microglia using Calcein-AM and PI. Microglia were seeded at 5×10^4 cells/well and cultured in mN3 medium for 24 and 48 h in both the presence and the absence of 10 ng/mL HLP. Green shows Calcein-AM-stained living cells and red shows PI-stained dead cells. Scale bars, 100 µm. (B) Quantification of percent survival of microglia. Survival was defined as living cells/total cells. (C) Quantification of proliferation of microglia. Proliferation was defined as total cells (both living and dead cells) cultured for 48 h. Experiments were done in triplicate and results are shown as mean ± S.E. The effect of HLP on survival and proliferation under this condition was not significant.

shown). Furthermore, the iNOS level decreased 1/7-fold by HLP-stimulation when the macrophage was under the treatment with LPS (data not shown). These mean that HLP shows diverse effects in iNOS production between macrophage cell line and microglia.

These results predict that HLP enhances inflammatory process in microglia.

DISCUSSION

In the nervous system, neuronal survival and/or neurite extension-promoting activities of HLP for sensory, sympathetic and motor neurons were revealed (5, 6, 24, 26). While the in vivo expression of Ron in microglia of multiple sclerosis patients and mouse model was reported previously (29), any function of

Table 1 *HLP modulates cytokine mRNA in microglia*

Gene	Induction ratio
IL-1α	6.4 ± 0.5
IL-1β	80.1 ± 4.5
IL-6	246.7 ± 14.6
TNFα	2.8 ± 0.6
GM-CSF	221.5 ± 20.5
iNOS	156.5 ± 10.6
BDNF	1.14 ± 0.016
CNTF	1.07 ± 0.04
HGF	0.22 ± 0.003
HLP	0.66 ± 0.2
Ron	1.01 ± 0.014

The amounts of mRNA in control were defined as 1.0, and the levels of indicated mRNA in microglia treated with HLP were calculated in relative value. Experiments were done in triplicates and results are shown as mean ± S.E.

HLP on microglia had not been defined. This study found the expression of the Ron/HLP receptor in murine primary microglia, which led us to reveal that HLP has the potential to stimulate microglial migration and increase mRNA levels of inflammatory cytokines, including IL-6 and GM-CSF, and inflammatory enzyme, iNOS in the cells. These findings are reminiscent of the involvement of HLP in activation of microglia in the brain.

In response to various neurodegenerative diseases and injuries, microglia proliferate and are recruited to the affected region, where they undergo morphological, immunophenotypical and functional changes (13, 14, 19). During these activation processes in microglia, expression of immunomodulatory cytokines and release of an inflammatory mediator, nitric oxide, are enhanced, and then microglia acquire phagocytotic properties. Similarly, the results from the present study show that HLP stimulates production of inflammatory cytokines and iNOS in primary microglia (Table 1). The in vivo states of microglia just before becoming phagocytotic may be formed with HLP.

The present study shows that HLP was able to promote microglial migration (Fig. 2). It took 18–24 h after HLP-stimulation to observe enhanced migration of microglia, which is much longer than the time required for migration enhanced by adenosine phosphate derivatives, such as ATP or ADP (A study conducted in a similar manner as the present one concluded that ATP and ADP induce microglial migration within 90 min) (11). Similarly, actin reorganization underlying the cell migration could not be observed within 30 min after HLP-stimulation in our experiments (data not shown), whereas the actin reorganization and the subsequent cell membrane ruffling were observed within 5 min following ATP and ADP-stimulation (11). It is possible that HLP-dependent protein synthesis or the secretion of some motogen following HLP-stimulation is required to induce microglial migration by HLP. More importantly, HLP and adenosine derivatives may share the roles in distinct physiological events that enhance microglial migration.

mRNA for IL-1α, IL-1β, IL-6, TNFα, GM-CSF and iNOS were upregulated by HLP-treatment in microglia (Table 1), although mRNA levels of BDNF, CNTF, HGF, and HLP were unchanged or changed little. IL-1α, IL-1β, IL-6, and TNFα are all proinflammatory cytokines induced at an early phase of inflammation. GM-CSF controls granulocyte and macrophage population and has the potential to activate microglia (2, 23). iNOS is an intracellular mediator of inflammation, catalyzing nitric oxide synthesis. These findings suggest that HLP enhances the early phase of inflammation in the brain through the secretion of these factors from microglia. In contrast to the function of HLP on the peripheral macrophages that suppresses inflammatory response of the cells (3, 16, 30), these data suggest that HLP has an ability to enhance inflammatory actions of microglia in the nervous system.

Alternatively, a part of cytokines upregulated by HLP elicit neuroprotective and neurotrophic functions. The microglial conditioned medium could support the survival of mesencephalic neurons isolated from the rat embryo (17). Activated microglia could protect neuronal cell line from hydrogen peroxide toxicity by release of IL-1α and IL-6 (1). Furthermore, among the cytokines upregulated by HLP, GM-CSF exhibits neurotrophic activity against NGF-dependent sympathetic neurons (9), and costimulation by TNFα and IL-1β enhances neurotrophic factor production in astrocytes (28). Therefore HLP may play neuroprotective roles through the upregulation of these cytokines from microglia.

Our present studies predict that HLP is one of the key players during neural degeneration and regeneration through the activation of cytokine production and cell migration in microglia. The mechanisms for in vivo activation of microglia are still elusive, however, the results of the present study propose novel functions of HLP in CNS and demonstrate the possibility that HLP regulates degeneration and regeneration of CNS neurons not only by direct association with neuronal cells but also in an orchestrated manner with the activation of microglia.

Further studies in this regard are necessary to shed light on the distinct biological significance of HLP in protective and degenerative processes in the CNS.

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