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HEPATOCYTE GROWTH FACTOR SPECIFICALLY EXPRESSED IN MICROGLIA ACTIVATED RAS IN THE NEURONS, SIMILAR TO THE ACTION OF NEUROTROPHIC FACTORS

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Summary: Hepatocyte growth factor (HGF) mRNA and its receptor (c-Met) mRNA were detected in the fetal and adult rat brain. Expression of c-Met mRNA was increased after birth. HGF mRNA was preferentially expressed in the microglia of the rat brain, while c-Met mRNA was expressed in neurons as well as astrocytes and microglia. Most of the neurons were c-Met positive, and HGF stimulated tyrosine phosphorylation of c-Met (140-kDa) in the neurons. HGF as well as bFGF also activated Ras in the neurons. These results suggest that HGF plays a biological role as one of the neurotrophic factors in the brain.

Hepatocyte growth factor (HGF), which was first found as a factor stimulating the DNA synthesis in hepatocytes (1), has been shown to be a pleotropic factor that acts as a motogen and morphogen for various epithelial cells in addition to being a mitogen (2-4). The HGF receptor is a transmembrane protein of 190-kDa, which consists of a 50-kDa extracellular α-subunit and 140-kDa β-subunit with a tyrosine kinase domain (5). Recently, the proto-oncogene c-*met* product has been identified as the β-subunit of the HGF receptor (6,7). The tyrosine of the c-*met* product is rapidly phosphorylated in response to HGF treatment (6,8). c-Met is detected in hepatocytes, keratinocytes and epithelial cells in the gastro-intestinal system, and also present in some other cells in normal human adult peripheral tissues.

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HGF mRNA is detected in rat brain as well as in lung and liver (9). HGF and c-Met protein are also immunohistochemically detected in human brain (10,11). However, the biological significances of HGF and c-Met have not yet been clarified in the brain. P19 EC cells that differentiate into neuronal cells in response to retinoic acid, are good model system for neuronal differentiation (12). Recently, Yang *et al.* reported that some neurons differentiated from P19 EC cells were c-Met positive (13). These results suggest that HGF is involved in the neuronal differentiation or some biological functions in neurons.

To clarify the biological roles of HGF and c-Met in the brain, we examined the developmental changes of HGF and c-Met mRNA and the cells expressing them. We also examined signal transduction of HGF in the neurons and discussed the biological roles of HGF and c-Met in the brain.

Materials and Methods

Isolation of neurons, astrocytes and microglia from rat embryonal brain

Wistar rats were obtained from Culea Japan (Tokyo). Cortical neuronal cultures were prepared from the brains of rat embryos at ED17 as described previously (14). The dissociated cells were cultivated in serum-free MEM to prevent proliferation of glial cells and maintained for another 3 or 4 days. Astrocytes were prepared from the cerebral hemispheres of newborn rat brain and cultivated in DMEM containing 10 % FCS for about 10 days. After neurons, microglia and oligodendrocyte were excluded by shaking overnight and washing three times, the astrocytes were transferred to new flask and maintained in DMEM containing 10 % FCS. Microglia were prepared according to the procedure developed by Nakajima et al. (15).

Assay of Ras activation

Neurons were labelled with 50 mCi/ml (1.85 MBq/ml) [^{32}P]Pi in Pi-free MEM for 3 hr at 37°C. The cells were treated with 10 ng/ml human recombinant bFGF (Mallinckrodt, Paris, KY) or rat recombinant HGF (Toyobo, Tokyo) for 5 min, and then they were washed twice with TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4) and solubilized with high Mg^{2+} buffer (20 mM Tris-HCl (pH7.5), 150 mM NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, and 0.5 % TritonX-100). After centrifugation, Ras was immunoprecipitated from the supernatant by anti-Ras antiserum, Y13-259, and the bound guanine nucleotides were analyzed according to the previously described method (16).

Immunoprecipitation and phosphorylation

For detection of phosphorylation of c-Met, the cells were treated with 10 ng/ml bFGF or HGF for 5 min. The cells were washed with TBS and then solubilized with 0.5 ml per dish of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_3VO_4, 1 mg/ml antipain, 1 mg/ml leupeptin, and 1 % TritonX-100). Extracts were centrifuged at 15,000 x g for 10 min and the supernatants were collected. The cell extracts were incubated with anti-c-Met immune complex containing anti-mouse c-Met antibody (SP260, Santa Cruz), goat anti-mouse IgG antibody (Cappel, West Chester, PA), and protein A-Sepharose CL4B (Pharmacia, Uppsala, Sweden) at 4°C overnight. The immune complex was washed with 0.3 ml of lysis buffer three times, treated with sample buffer, and then subjected to 6% polyacrylamide gel electrophoresis. Proteins in the gel were electrophoretically blotted to a nitrocellulose filter. For detection of phosphotyrosine, the filter was incubated with anti-phosphotyrosine antibody (Py20, Seikagaku Co., Tokyo) and then with anti-mouse IgG antibody conjugated with alkaline phosphatase (Promega, Madison,WI).The imunoreactivities were visualized by incubating the filter in the reaction buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$, 40 mM 5-bromo-4-chloro-3-indolylphosphate, and 80 mM nitro blue tetrazolium salt) at room temperature.

Immunostaining

Neurons isolated from rat ED 17 were fixed with 2 % paraformaldehyde at 4 °C for 20 min. After the cells were washed with PBS, they were incubated with mouse anti-

MAP-2 monoclonal antibody (Sigma, St Louis, MO) and rabbit anti-c-Met antibody at 37 °C for 2 hr. Their immunoreactivities were visualized by affinity-purified rhodamine-labelled goat $F(ab')^2$ anti-mouse immunoglobulin and FITC-labelled goat $F(ab')^2$ anti-rabbit immunoglobulin (TAGO. Co. Burlingame, CA.), respectively. Specificity of the immunostaining was examined by replacement of the primary antibodies with control mouse myeloma immunoglobulin and preimmune rabbit serum.

Northern blot analysis

Total RNA was prepared from neurons, astrocytes and microglia cells according to the acid guanidium thiocyanate-phenol-chloroform (AGPC) method (17). Twenty micrograms of total RNA was subjected to formalin agarose gel electrophoresis and blotted to a nylon filter (Amersham, Buckinghamshare, England). The filters were hybridized with [^{32}P]-labelled probes of rat HGF and rat *c-met* cDNA, which were prepared from rat embryonal tissues by reverse transcriptase PCR and whose DNA sequences were identified by a fully automatic DNA sequencer (Pharmacia, Milwaukee, WI).

Results and Discussion

HGF is secreted from various cells and has pleotropic effects on the growth and motility of various epithelial and endothelial cells such as hepatocytes, melanocytes, keratinocytes (18) and renal tubular cells (19). In the liver, HGF is expressed in non-parenchymal cells (20). In the human brain, the distributions of the HGF and c-Met have been shown by immunohistochemical staining (10,11). To clarify

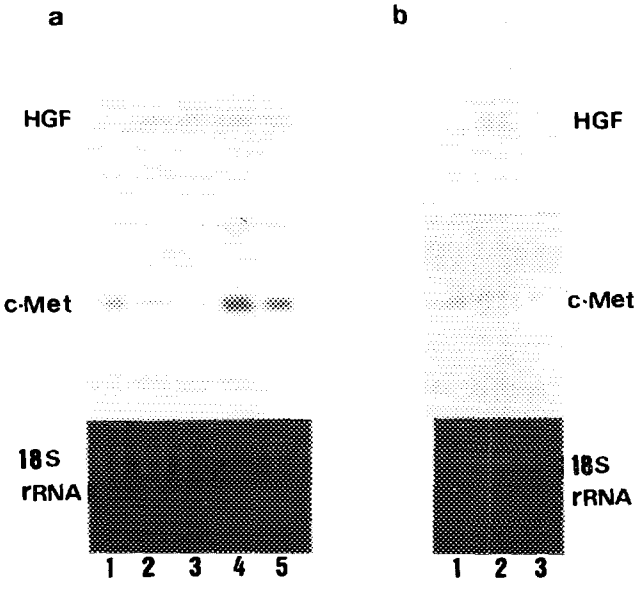

Figure 1.
Detection of HGF and c-Met mRNA in the rat brain during development and the cell population in the brain.
a) The developmental changes in the expression of HGF and c-Met mRNA in the rat brain. Lane 1, ED14; lane 2, ED16; lane 3, ED18; lane 4, newborn; lane 5, adult (8-week old). b) Detection of HGF and c-Met mRNA in various cell populations of the rat brain by northern blot analysis. Lane 1, astrocytes; lane 2, microglia; lane 3, neurons.

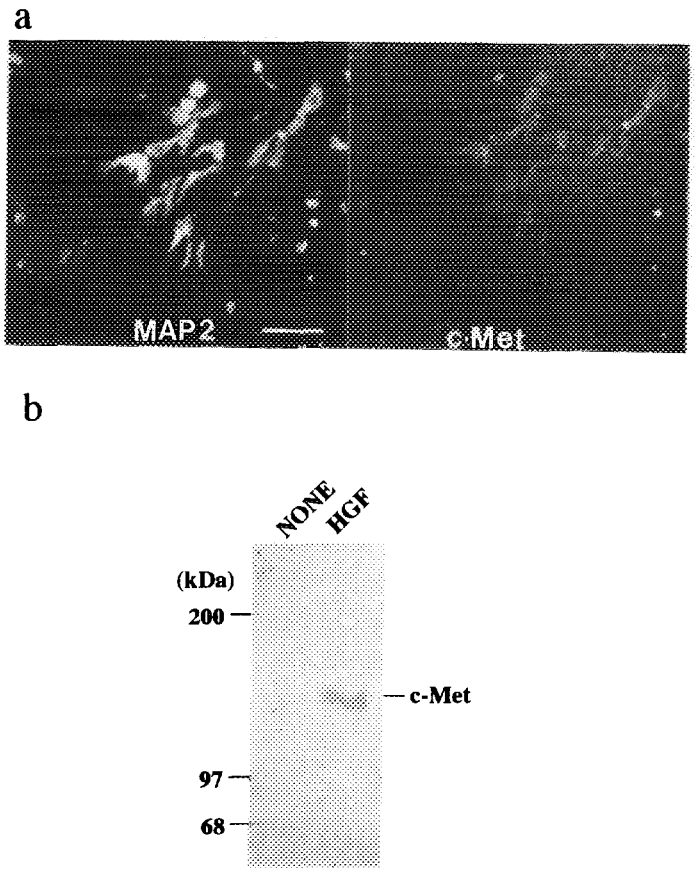

Figure 2.
The effects of HGF on tyrosine phosphorylation of c-Met in neurons of ED17 rat brain. (a) Double immunostaining of neurons obtained from ED17 rat brain with anti-MAP-2 and anti-c-Met antiserum. The bar indicates 0.1 mm. (b) Tyrosine phosphorylation of c-Met in the neurons of ED17 rat brain. Neurons obtained from ED17 rat brain were treated with or without HGF (10 ng/ml) for 5 min. c-Met in the extracts was immunoprecipitated, and its tyrosine phosphorylation of c-Met was detected by anti-tyrosine phosphate specific antiserum.

the biological roles of HGF in the brain, we examined the developmental changes of HGF and c-Met mRNA and identified the cells expressing them.

HGF and c-Met mRNA were detected in the brain at ED14, and their amounts showed little change during development (Figure 1a). After birth, the amount of c-Met mRNA increased. The expression of HGF and c-Met mRNA were examined in neurons, astrocytes and microglia (Figure 1b). HGF mRNA was selectively expressed in the microglia. There is a discrepancy between our results and those of a previous study on the cells expressing HGF in the brain; HGF protein was mainly detected in the astrocytes of human brain by immunohistochemical staining (11), while HGF mRNA was mainly detected in microglia in the present study and was slightly detected in the astrocytes.

One of the reasons for this discrepancy may be due to the detection method or species used. The discrepancy may also be due to the developmental stages of the sample used. Since HGF mRNA was expressed in the brain at ED14-16, when microglia does not appear in the brain, we can not exclude the possibility that neuronal precursor cells express HGF in the fetal brain. HGF, acting as the motogen, may be involved in the migration of microglia and neuronal cells into the brain. The cells expressing HGF in the developing brain remain to be identified.

It is of interest that c-Met mRNA was detected in the neurons as well as the astrocytes and microglia. Double immunostaining showed that c-Met was distinguishably positive in most of the MAP-2-positive neurons isolated from rat brain at ED17 (over 95% of the population was MAP-2 positive) (Figure 2a). The c-Met-

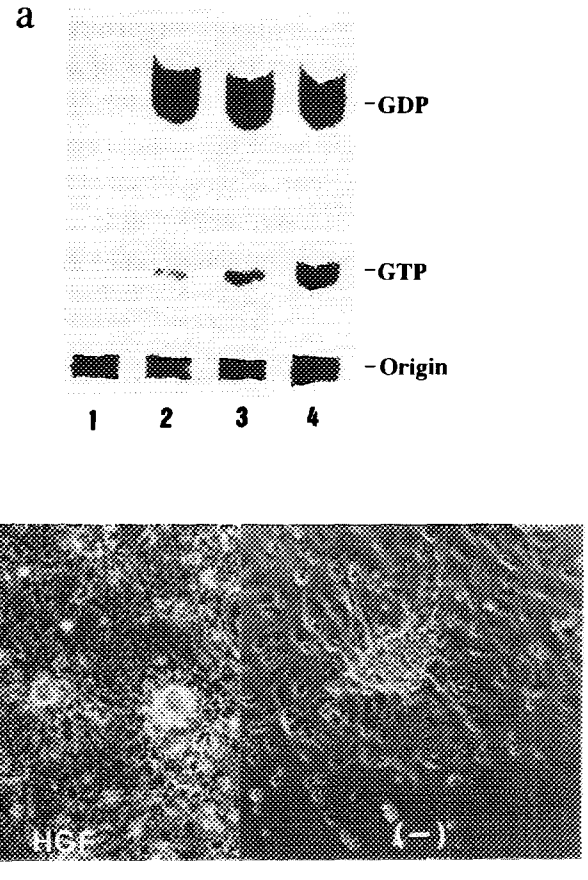

Figure 3.
Activation of Ras in the neurons by HGF.
Activation of Ras in the neurons by HGF and bFGF. Neurons were labelled with [^{32}P] orthophosphate for 3 hr. After the neurons were treated with HGF or bFGF for 5 min, Ras was immunoprecipitated, and the bound guanine nucleotides were analyzed by thin layer chromatography and autoradiography (a). Lanes1 and 2, untreated cells; lane 3, HGF-treated cells; lane 4, bFGF-treated cells. Lane 1, without anti-Ras antiserum; lanes 2-4, with anti-Ras antiserum. (b) Neurite outgrowth inducing effect of HGF. Fetal rat brain (ED 17) cortex fragment was incubated with or without HGF (20 ng/ml) for 2 days.

positive cells were not detected in the MAP-2-negative cells. HGF induced tyrosine phosphorylation of a 140-kDa protein in neurons that was recognized by the anti-c-Met antibody (Figure 2b). Thus HGF stimulated tyrosine phosphorylation of c-Met in some of the neurons.

Neurotrophic factors such as NGF, BDNF and NT-3 and bFGF have tyrosine-kinase-type receptors like HGF (21). The downstream of the signal transduction from their receptors is Ras (22,23). Recently, activation of Ras has been also shown to be the downstream of the activation of the *c-met* product (24). We examined whether HGF activates Ras in neurons or not. The basal level of GTP-bound Ras was 6.1±0.1% in the untreated neuronal cells. However, the treatment with HGF and bFGF for 5 min elevated the amount of GTP-bound Ras to 9.2±0.2% and 18.7±0.4%, respectively (Figure 3a).

Thus HGF stimulated tyrosine phosphorylation of its receptor and activated Ras in the neurons like neurotrophic factors do. Neurotrophic factors including bFGF are known to be secreted from microglia. These results suggest that HGF has effects on the neurons as one of the neurotrophic factors secreted from microglia by the paracrine system. However, we could not detect the mitogenic effects of HGF on the neurons, while we detected the neurite outgrowth inducing effects of HGF on the neurons of the fetal brain (Figure 3b) and the RA-treated P19 EC cells (unpublished observations). Thus HGF may be involved in the neuronal differentiation during development.

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