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ScienceDirect
Biochemical and Biophysical Research Communications 366 (2008) 110–116
BBRC
www.elsevier.com/locate/ybbrc

Inhibition of tumor–stromal interaction through HGF/Met signaling by valproic acid

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Received 16 November 2007
Available online 11 December 2007

Abstract

Hepatocyte growth factor (HGF), which is produced by surrounding stromal cells, including fibroblasts and endothelial cells, has been shown to be a significant factor responsible for cancer cell invasion mediated by tumor–stromal interactions. We found in this study that the anti-tumor agent valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, strongly inhibited tumor–stromal interaction. VPA inhibited HGF production in fibroblasts induced by epidermal growth factor (EGF), platelet-derived growth factor, basic fibroblast growth factor, phorbol 12-myristate 13-acetate (PMA) and prostaglandin E_2 without any appreciable cytotoxic effect. Other HDAC inhibitors, including butyric acid and trichostatin A (TSA), showed similar inhibitory effects on HGF production stimulated by various inducers. Up-regulations of HGF gene expression induced by PMA and EGF were also suppressed by VPA and TSA. Furthermore, VPA significantly inhibited HGF-induced invasion of HepG2 hepatocellular carcinoma cells. VPA, however, did not affect the increases in phosphorylation of MAPK and Akt in HGF-treated HepG2 cells. These results demonstrated that VPA inhibited two critical processes of tumor–stromal interaction, induction of fibroblastic HGF production and HGF-induced invasion of HepG2 cells, and suggest that those activities serve for other anti-tumor mechanisms of VPA besides causing proliferation arrest, differentiation, and/or apoptosis of tumor cells.

Keywords: Hepatocyte growth factor; Valproic acid; Histone deacetylase inhibitor; Butyric acid; Trichostatin A; Induction; Tumor invasion; Dermal fibroblast

Hepatocyte growth factor (HGF), also known as scatter factor, was originally discovered as a mitogenic factor of rat hepatocytes in primary culture [1–5]. HGF is now recognized as a pleiotropic factor that functions as a mitogen, motogen, morphogen, and anti-apoptotic factor acting on various types of cells [6,7]. Based on these actions, HGF has been shown to play critical roles in developmental and regenerative events of the liver and other tissues [8–11]. In addition to regulation of normal cell functions, many studies have shown that HGF is involved in malignant cell transformation and growth, invasion and metastasis in tumor cells [12,13]. HGF is mainly produced by surrounding stromal cells such as fibroblasts and endothelial cells and stimulates growth, metastasis, and/or invasiveness of cancer cells expressing the HGF receptor Met in a paracrine manner [14]. Thus, the HGF and Met pathway is one of the most commonly cited soluble factor signaling pathways in the

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.11.089

tumor–stromal interaction [14]. Clinical studies on hepatocellular carcinoma (HCC) have also suggested the involvement of interaction of HGF and c-Met in human tumor invasion and metastasis. High levels of serum HGF in patients with HCC are associated with tumor metastasis [15]. Therefore, inhibition of fibroblastic HGF production and HGF-induced aggressive behavior of tumor cells is expected to suppress proliferation, metastasis, and invasiveness of malignant tumor cells, including HCC cells.

Acetylation and deacetylation of nucleosomal core histones play an important role in the modulation of chromatin structure and the regulation of gene expression. The disruption of balance between histone acetyltransferases and histone deacetylases (HDACs) has been suggested to be associated with cancer development. HDAC activity is increased in cancer cells and has been linked to carcinogenesis [16]. Indeed, it has recently been shown that global hypo-acetylation of histone H4 is a common feature of human tumor cells [17]. Valproic acid (VPA), an effective anticonvulsant in the treatment of epilepsy, as well as butyric acid (BA) inhibits the activity of zinc-dependent class I and class II HDACs [18]. HDAC inhibitors induce proliferation arrest, differentiation, and/or apoptosis of tumor cells but not of normal cells [17]. Based on these activities, HDAC inhibitors have exhibited anti-tumor effects in clinical trials [17,19]. However, the exact mechanisms by which HDAC inhibitors exert an anti-tumor effect and modulate gene expression are not completely understood and remain a subject of intense investigation.

In the present study, we investigated whether VPA affects the HGF and Met pathway in the tumor–stromal interaction and we found that VPA potently inhibited two processes of the interaction, induction of fibroblastic HGF production and HGF-induced invasion of HepG2 HCC cells. Our results suggest that VPA exerts anti-tumor effects at least partly through the inhibition of tumor–stromal interaction which may constitute a new class of targets for chemoprevention of tumor invasion.

Materials and methods

Cell culture. Human dermal fibroblasts derived from 200 individual neonatal donors (Cell Systems, Kirkland, WA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air as described previously [20]. HepG2 cells were obtained from Tohoku University (Sendai, Japan) and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Determination of HGF production. The medium of confluent fibroblasts cultured in 96-well plates (Nunc, Roskilde, Denmark) was replaced with the fresh medium described in the previous section or that containing HDAC inhibitors, and the cells were preincubated for 1 h. HGF inducers were then added, and the conditioned medium was collected after being incubated for various periods. The sandwich ELISA for human HGF was performed at room temperature as described previously [21], with slight modification [22].

MTT assay. Confluent fibroblasts were incubated with HDAC inhibitors and HGF inducers as described in the previous section. HepG2 cells (5×10^4 cells/0.2 ml/well) seeded in 96-well plates (Nunc) were preincubated for 1 h with or without VPA and incubated for 24 h with or without HGF in the presence or absence of VPA. The medium was then replaced with 100 μl of the same fresh medium, and the cultures were incubated for 1 h. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was performed as described previously [23].

Northern blot analysis. The medium of confluent fibroblasts grown in 90-mm dishes (Nunc) was replaced with the same fresh medium, and the cells were incubated for about 15 h. VPA or trichostatin A (TSA) was added without a medium change, and the cells were preincubated for 1 h. The HGF inducer was then added. After being incubated for 15 or 40 h, total RNA was isolated from the cells using RNA Bee (TEL-TEST, Friendswoods, TX). Northern blotting was performed as described previously [20].

Real-time PCR analysis. The medium of confluent fibroblasts cultured in 6-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for 24 h. After treatment with or without cycloheximide for 1 h, the cells were incubated for 1 h with or without VPA and then for an additional 8 h with or without phorbol 12-myristate 13-acetate (PMA). Total cellular RNA was isolated as described above. After treatment with DNase, first-strand cDNA synthesis from 0.5 μg RNA was performed using reverse transcriptase with random and oligo-dT primers. Real-time PCR was performed with a LightCycler (Roche, Indianapolis, IN) using SYBR® Green Real-time PCR Master Mix (Toyobo Co., Osaka, Japan) according to the manufacturer's protocol. The nucleotide sequences of primers for HGF were as follows: forward, 5′-CAATAGCATGTCAAGTGGAG-3′; reverse, 5′-CTGTGTTCGTGTGGTATCAT-3′ (amplicon size: 180 bp). The nucleotide sequences of primers for 28S rRNA used as an internal control were: forward, 5′-GTTCACCCACTAATAGGGAACG-3′; reverse, 5′-GGATTCTGACTTAGAGGCGTTC-3′ (amplicon size: 213 bp). The PCR conditions were as follows: HGF, 1 cycle of 95 °C for 30 s followed by 60 cycles of 95 °C for 5 s, 57 °C for 0 s, and 72 °C for 25 s; 28S rRNA, 1 cycle of 95 °C for 30 s followed by 55 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 15 s. Relative cDNA copy numbers were computed on the basis of data with a serial dilution of a representative sample for each target gene.

Western blot analysis. The medium of subconfluent HepG2 cells grown in 24-well plates (Nunc) was replaced with the same fresh medium, and the cells were incubated for about 15 h. VPA was added without a medium change, and the cells were preincubated for 1 h. Then, HGF was added. After being incubated for an appropriate period, the cells were harvested, and Western blotting was performed as described previously [24]. In some experiments, cytosolic and nuclear extracts of the cells in 6-well plates (Nunc) were prepared according to the manufacturer's instructions (Active Motif, Carlsbad, CA).

Cell invasion assay. The *in vitro* invasion activities were examined as reported previously [25]. Polycarbonate membranes with 8-μm pores of Transwell® inserts (Corning, New York), upper culture chambers, were coated with 50 μl of growth factor-reduced Matrigel™ (BD Biosciences, San Jose, CA) in cold RPMI 1640 medium (0.25 mg/ml) and dried overnight. HepG2 cells suspended in RPMI 1640 medium supplemented with 2% FBS were seeded onto the upper culture chambers at a density of 1.5×10^5 cells/cm^2 (0.2 ml/well), whereas the lower culture chambers of 24-well plates were each filled with 0.8 ml of serum-free RPMI 1640 medium containing HGF, VPA or the combination of HGF and VPA. After the incubation for 24 h, the cells on the upper surface of the membrane were wiped off with a cotton swab. The cells that had invaded the lower surface of membranes were fixed for 10 min with methanol, stained with Giemsa solution overnight, and counted under a microscope.

Statistical analysis. All results were expressed as means and SEM of several independent experiments. The data were analyzed by Dunnett's *t*-test, Dunnett's T3, Tukey's test or Student's *t*-test. *P* values less than 0.05 were regarded as significant.