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

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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

Introduction

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 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 37ºC 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 37ºC 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 Realtime 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 95ºC for 30 s followed by 60 cycles of 95ºC for 5 s, 57ºC for 0 s and 72ºC for 25 s; 28S rRNA, 1 cycle of 95ºC for 30 s followed by 55 cycles of 95ºC for 5 s, 60ºC for 5 s and 72 ºC 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.

Results

Inhibition by valproic acid and other HDAC inhibitors of HGF induction in human dermal fibroblasts

HGF expression has been demonstrated to be up-regulated in stromal cells by tumor cell-secreted soluble growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [26]. Therefore human dermal

fibroblasts were incubated for 72 h with VPA in the presence and absence of suboptimal doses of those growth factors. HGF secreted from the cells was then determined by an HGF ELISA. The effect of VPA on basal HGF production could hardly be determined because of the small amount of HGF produced (data not shown). VPA significantly inhibited EGF-, PDGF- and bFGF-induced HGF production with IC_{50} values of 0.15, 0.19 and 0.17 mM, respectively (Table 1). HGF production stimulated by other inducers, PMA, 8-bromo-cAMP and prostaglandin E_2 (PGE_2), was also inhibited by VPA with IC_{50} values of 0.86, 0.48 and 0.12 mM, respectively (Table 1). The number of viable cells in the cultures treated with or not treated with PMA and 8-bromo-cAMP was not affected by any concentration of VPA as determined by the MTT method (Fig. 1A and 1B). On the other hand, VPA inhibited the EGF-induced increase in number of viable cells with IC_{50} of 0.42 mM and up to the level in control cultures at 2 mM (Fig. 1C), whereas cell viability, determined by the Trypan Blue-exclusion test, in the cultures treated or not treated with EGF was not decreased by VPA (data not shown).

Next we examined whether other HDAC inhibitors modulate HGF induction in human dermal fibroblasts. While BA as well as VPA inhibits class I and class IIa members of the HDAC family, TSA inhibits broad-spectrum HDACs [18]. Both BA (2 mM) and TSA (1 μM) also significantly inhibited HGF production induced by EGF, PMA, 8-bromo-cAMP and PGE_2 (data not shown).

EGF- and PMA-induced HGF production is accompanied by up-regulation of HGF gene expression [26,27]. Effects of VPA and TSA on HGF gene expression up-regulated by PMA or EGF are shown in Fig. 2A and 2B. VPA significantly inhibited PMA- and EGF-induced HGF mRNA expression by 32% and 81%, respectively. TSA inhibited PMA- and EGF-induced HGF mRNA expression by 90% and 61%, respectively.

Inhibition of HGF gene up-regulation by VPA is not blocked by cycloheximide treatment.

HDAC inhibitors generally activate expression of many genes, but the expression of some genes, including estrogen receptor α, is down-regulated by histone acetylation induced by HDAC inhibitors [28]. Since the down-regulation in most cases is prevented by concomitant treatment

with cycloheximide, reduction of gene expression by HDAC inhibitors is presumably dependent on the synthesis of transcriptional repressors [28]. Thus, we tested whether VPA-caused repression of HGF gene up-regulation induced by PMA is such a case. As shown in Fig. 2C, treatment with cycloheximide did not prevent VPA-caused reduction of HGF gene up-regulation.

Inhibition of HGF-induced invasion of HepG2 cells by valproic acid

Tumor-stromal interaction through HGF/c-Met signaling plays an important role in growth, invasion, and metastasis of tumor cells. It has been shown that HGF stimulates the invasiveness of the human hepatoblastoma cell line HepG2 in a Matrigel cell invasion assay [29,30]. To investigate whether VPA affects the cell invasion induced by HGF, HepG2 cells were incubated with HGF, VPA or the combination of HGF and VPA for 24 h. The number of cells that invaded the filters coated with Matrigel basement membrane matrix were then counted. HGF strongly induced the invasion of HepG2 cells, as reported previously [29,30] (Fig. 3A and 3B). Importantly VPA significantly suppressed HGF-induced invasion of HepG2 cells (Fig. 3A and 3B), whereas the number of viable cells, determined by the MTT assay, in the cultures treated with HGF was not decreased by VPA (Fig. 3C). BA (2 mM) and TSA (1 μM) also significantly inhibited HGF-induced invasion of HepG2 cells (data not shown).

It has been suggested that HGF induces cell invasion of HepG2 cells via the ERK pathway and phosphatidylinositol 3-kinase (PI3K)-Akt pathway [30,31]. VPA (2 mM), however, did not show any significant effect on phosphorylation of ERK and Akt or nuclear translocation of phosphorylated ERK in the cells treated for 15, 30 and 60 min with HGF (10 ng/ml) (data not shown).

Discussion

Enhancement of cancer cell invasion by stromal fibroblasts has been demonstrated in a variety of cancers, including breast, gallbladder, esophageal and prostate cancers [32]. HGF, which is

mainly secreted 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 [14]. Elevated levels of tumor and plasma HGF have been observed in a number of cancers such as lung, breast, prostate, and hepatocellular cancers [33]. We demonstrared in this study that VPA and other HDAC inhibitors blocked tumor-stromal interactions at two steps: inhibition of HGF induction in human dermal fibroblasts and suppression of HGF-induced HepG2 cell invasion. Thus, HDAC inhibitors have other antitumor mechanisms besides causing proliferation arrest, differentiation, and/or apoptosis of tumor cells.

HGF expression has been demonstrated to become up-regulated in stromal cells by tumor cell-secreted soluble cytokines and growth factors, including IL-1α, IL-1β, EGF, PDGF, and bFGF [26,27]. PGE_2, which is implicated in the proliferation and invasiveness of tumor cells [30], also induces HGF production in fibroblasts [20]. Induction of HGF by EGF, other growth factors and PGE_2 was more sensitive to VPA than was HGF induction by other inducers, the IC_{50} being approximately 0.15 mM. This concentration is clinically achieved in plasma of patients treated with VPA for epilepsy, *e.g.*, a mean trough plasma level of 0.44 mM after 3-month treatment with an average daily dose of 720 mg [34]. The values in the MTT assay of cultures of human dermal fibroblasts and HepG2 cancer cells were not decreased by treatment with VPA except for those in EGF-stimulated human dermal fibroblasts. In accordance with the known antiproliferative activity of the short-chain fatty acid, VPA decreased the values in the MTT assay induced by EGF up to those of control cultures not treated with EGF. In addition, VPA did not decrease cell viability, determined by the Trypan Blue-exclusion test, in cultures treated or not treated with EGF. These findings collectively indicate that VPA is not cytotoxic to the cells.

Histone acetylation causes alteration of nucleosomal conformation and thus increases the accessibility of transcriptional regulatory proteins to chromatin templates and subsequent transcription [35]. These changes are thought to be one mechanism by which HDAC inhibitors generally activate expression of many genes. Nevertheless, the expression of some genes, including estrogen receptor α gene, the oncogene MYC and tumor suppressor gene p53, is down-regulated by histone acetylation induced by HDAC inhibitors [28]. Since VPA- and

TSA-caused reduction in expression of most of those genes is blocked by concomitant treatment with cycloheximide, down-regulation of those genes by HDAC inhibitors is presumably dependent on the synthesis of transcriptional repressors [28]. Inhibition of up-regulated HGF gene expression by VPA, however, was not prevented by cycloheximide and was thus not such a case.

VPA potently inhibited HepG2 cell invasion induced by HGF without suppressing cell proliferation. Cell invasion is a major event involved in the complex multistep process of tumor metastasis. Invasion of tumor cells requires destruction of basement membranes, proteolysis of extracellular matrix (ECM), pseudopodial extension, and cell migration [36]. After modifying the ECM barrier, tumor cells migrate through the barrier and proliferate at a secondary site. HGF is one of the potent motility factors and contributes to metastasis by stimulation of motility [12]. Activation of PI3K-Akt and ERK pathways has been suggested to be critical for cell motility stimulated by HGF [30,31,37], but phosphorylation of neither Akt nor ERK in HepG2 cells stimulated with HGF was reduced by treatment with VPA. Although its mechanism remains to be investigated, inhibition of tumor cell invasion by HDAC inhibitors may constitute a new class of strategies of chemoprevention of tumor metastasis.

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Figure legends

Figure 1. VPA has no cytotoxic effects on fibroblasts treated with or without PMA and 8-bromo-cAMP and inhibits EGF-induced cell proliferation. Confluent cells were preincubated for 1 h with the indicated concentrations of VPA and then incubated for 24 h with or without 3 nM PMA and for 72 h with or without 0.3 mM 8-bromo-cAMP or 3 ng/ml of EGF in the presence or absence of VPA. The number of viable cells was measured by the MTT method. The data are means ± SEM of three independent experiments. *$P < 0.05$, ***$P < 0.001$, as compared with the values of medium alone or respective inducer alone.

Figure 2. VPA- and TSA-caused inhibition of HGF gene expression up-regulated by PMA or EGF and effects of cycloheximide on VPA-caused inhibition. (A, B) Confluent fibroblasts were preincubated for 1 h with or without 2 mM VPA or 1 μM TSA and then incubated for 15 h with or without 3 nM PMA or for 40 h with or without 3 ng/ml of EGF in the presence or absence of VPA or TSA. The signal intensity of the 6.4-kb HGF mRNA band in the autoradiograms was normalized to the fluorescence intensity of the 28S rRNA band, and results are expressed as relative levels to the value of untreated cells. The data are means of three independent experiments. Bars indicate SEM. **$P < 0.01$, ***$P < 0.001$, as compared with the values of medium alone or respective inducer alone. (C) Confluent fibroblasts were preincubated for 1 h with or without 1 μg/ml of cycloheximide (CHX) and then for 1 h with or without 2 mM VPA followed by incubation for an additional 8 h with or without 3 nM PMA. The expression levels of HGF mRNA and 28S rRNA were measured by real-time PCR. Results are expressed as relative levels to the value of untreated cells after being normalized to the 28S rRNA levels. The data are means of three independent experiments. Bars indicate SEM. *$P < 0.05$, **$P < 0.01$, as compared with the values of the respective PMA alone.

Figure 3. Inhibition of HGF-induced invasion of HepG2 cells by VPA. (A, B) HepG2 cells, seeded onto the upper chamber consisting of filters coated with Matrigel™, were preincubated for

1 h with or without of 2 mM VPA added to the lower chamber and then incubated for 24 h with or without 10 ng/ml of HGF added to the lower chamber. Cells that invaded the filter were visualized and counted microscopically at × 200 magnifications. Scale bar=100 μm. (C) HepG2 cells seeded in 96-well plates were preincubated for 1 h with or without 2 mM VPA and incubated for 24 h with or without 10 ng/ml of HGF in the presence or absence of VPA. The number of viable cells was then measured by the MTT method. The data are means of four (invasion) or three (MTT) independent experiments. Bars indicate SEM. $*P < 0.05$, as compared with the values of medium alone or HGF alone. NS, not significant.