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Short communication

Transforming growth factor β receptor family ligands inhibit hepatocyte growth factor synthesis and secretion from astrocytoma cells

Naibedya Chattopadhyay[a,*], Jacob T-Felt Hansen[a], Madan M. Godbole[b], Edward M. Brown[a]

[a] *Division of Endocrinology, Diabetes and Hypertension and Membrane Biology Program, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, Rm 205, Boston, MA 02115, USA*
[b] *Department of Endocrinology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow 226 014, India*

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Abstract

Transforming growth factor β (TGFβ) and hepatocyte growth factor (HGF) promote glioma progression. Using U87human astrocytoma cells, which express TGFβ receptors (TβRs), we show (1) mRNA expression of Smads (2, 3, 4), bone morphogenetic protein (BMP)- and activin-A receptors; (2) TGFβ1 inhibits and HGF induces proliferation; (3) TGFβ1 and activin-A equipotently inhibit HGF secretion more than BMP-2, but none alters c-Met expression. Because interfering with TβR signaling might nullify the beneficial inhibition of HGF secretion, activin-A should instead be considered for combination glioma therapy.

Theme: Disorders of the nervous system
Topic: Neuro-oncology

Keywords: Activin-A; BMP; Tumor; Proliferation; Angiogenesis

Transforming growth factor β (TGFβ) and hepatocyte growth factor (HGF) are multi-functional and pro-malignant cytokines that profoundly favor glioma progression. TGFβ belongs to a family of ligands consisting of bone morphogenetic proteins (BMPs), activins, and inhibins. Common signaling strategies utilized by these ligands involve type-I and -II serine-threonine kinase receptors with subsequent phosphorylation of Smad proteins (for review, see Refs. [11,35]). In glial cells, TGFβ varies from being a growth inhibitor of normal glial cells [29] to serving as a progression factor in glioblastomas [16,17,20,26,28]. Because of its tumor-promoting roles, TGFβ has become an experimental target for glioma therapy [13,33,41].

HGF, in addition to its neurotropic role [14], is also expressed in normal and malignant glial cells [12,37,19]. HGF, along with its receptor, c-Met, are expressed at abnormally high levels in gliomas [23,37,40] and exert strong proliferative and motogenic actions in an autocrine/paracrine manner. Hence, inhibition of HGF production and/or neutralization of its protein product are important potential chemotherapeutic strategies in the control of glioma growth [1,5].

The three isoforms of TGFβ (TGFβ-1, -2, and -3) are differentially expressed in gliomas, with TGFβ-2 being the most highly expressed. Furthermore, HGF induces TGFβ-2 in U87 cells [38]. Interactions between these two cytokines have been implied by reports of their complementary roles in mammary gland ductal morphogenesis [27] and antagonistic actions on chemotactic function in liver epithelial cells [34]. Moreover, since both TGFβ and HGF are overexpressed in gliomas and aid their malignant progression, we sought to study their effects on the proliferation of glioma cells (U87) and the effects of TGFβ-1 and its subfamily ligands on HGF secretion by U87 cells.

DMEM was purchased from GIBCO-BRL (Grand Island, NY), fetal bovine serum (FBS) from Hyclone (Logan, UT), the U87 cell line from the American Type Culture Collection (Manassas, VA), BrdU proliferation kit from Roche Diagnostics (Indianapolis, IN), SYBR green real-time PCR kit from Qiagen (Carlsbad, CA), and activin-A, BMP-2, TGFβ-1, and ELISA kits for HGF from R&D Systems (Minneapolis, MN). U87 cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

* Corresponding author. Tel.: +1-617-732-4093; fax: +1-617-732-5764.
E-mail address: Naibedya@rics.bwh.harvard.edu (N. Chattopadhyay).

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Table 1
PCR primer pairs for human BMP and activin receptors and Smads

Gene	Primer pairs
BMP-RIA	TAAAGGTGACAGTACACAGGAACA (F)
	TCTATGATGGCAAAGCAATGTCC (R)
BMP-RIB	TACAAGCCTGCCATAAGTGAAGAAGC (F)
	ATCATCGTGAAACAATATCCGTCTG (R)
BMP-RII	TCCTCTCATCAGCCATTTGTCCTTC (F)
	AGTTACTACACATTCTTCATAG (R)
Activin RIA	AAGATGAGAAGCCCAAGGTC (F)
	GCAGGCAGGCTAAAAGACAT (R)
Activin RIB	CTGGCTGTCCGTCATGATGCA (F)
	CAATTCGCTCTCAGAGTCTCC (R)
Activin RII	ACCAGTGTTGATGTGGATCTT (F)
	TACAGGTCCATCTGCAGCAGT (R)
Smad 2	ATCCTAACAGAACTTCCGCC (F)
	CTCAGCAAAAACTTCCCCAC (R)
Smad 3	AAGGACGAGGTCTGCGTGAATC (F)
	TTTTCGGGGATGGAATGGC (R)
Smad 4	GCATCGACAGAGACATACAG (F)
	CAACAGTAACAATAGGGCAG (R)

Subconfluent (70–80%) U87 cells grown on 96-well plates were serum-starved for 4 h in DMEM containing 0.2% BSA. Cells were incubated in the same medium with various concentrations of TGFβ-1 or HGF for 18 h, and proliferation was measured by BrdU incorporation according to the manufacturer's instructions. For the last 3 h of the 18-h stimulation period, the cells were pulsed with BrdU. Absorbance at 450 nm was measured with a microplate reader (Model 550, Bio-Rad, Hercules, CA).

mRNA expression of Smads 2, 3, and 4 as well as BMP and activin receptors were assessed by RT-PCR using 2.0 μg DNase-treated total RNA extracted from U87 cells as described previously [6]. Single-step RT-PCR was performed using a kit (Qiagen) and following the manufacturer's instructions; sequencing of the PCR products was performed with the respective primer pairs that were used for amplification of the products. Previously published primer pairs used are shown in Table 1 [25,39]. The temperature cycle protocol for 35 cycles was 30 s at 94 °C, 30 s at 56 °C (54 °C for BMP-RII), and 30 s at 72 °C (PCR system 9700, Applied Biosystems, Foster City, CA).

For Northern analysis of HGF, 3.0 μg poly A^+ RNA per sample was employed following a protocol described previously [6], using as the probe a ^{32}P-labeled 2.3 kilobase pair (kbp) fragment of human HGF (a generous gift from Dr. T. Nakamura, Osaka University School of Medicine, Japan).

To study the effect of various TGFβ receptor family ligands on HGF secretion, U87 cells were cultured and serum-deprived as outlined above. This medium was replaced by the same medium with growth factors added at varying concentrations, as described below. After an 18-h incubation with the cytokines, conditioned medium was assayed for HGF concentration using an ELISA kit (assay sensitivity 125 pg/ml). The HGF content of each well was normalized to cellular protein.

SYBR green chemistry was used to perform quantitative determination of c-Met and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA following a published protocol [36]. Primers were synthesized using Primer Express version 2.0.0 (Applied Biosystems), as follows: 5′ -GACAAATGTGTGCGATCGGAG-3′ (c-Met sense), 5′ -CATATGGTCAGCCT-TGTCCCTC-3′ (c-Met antisense); 5′ -CAGGGCTGCTTTTAACTCTGGTAA-3′ (GAPDH sense), 5′ -AATTTGCCATGGGTGGAATC-3′ (GAPDH antisense). cDNA library was synthesized with the Omniscript RT Kit (Qiagen) using 2 μg total RNA. Real-time PCR was performed using QuantiTech™ SYBR PCR (Qiagen) on ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

Results are expressed as mean ± S.E.M. Statistical evaluation for differences between group means was carried out using a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). For all statistical tests, values of $p < 0.05$ were considered to indicate a statistically significant difference between groups.

As U87 cells express c-Met [6], we studied the effect of HGF on their proliferation. We found that HGF induces proliferation of U87 cells by 43% (Fig. 1A). Stimulation of proliferation by HGF has been reported in primary

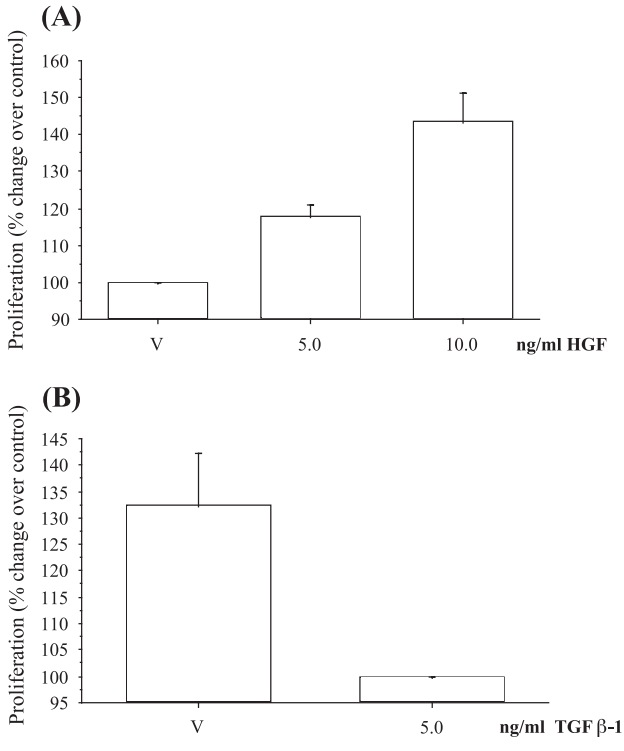

Fig. 1. (A) HGF induces proliferation of U87 cells. Subconfluent cells were serum-starved for 4 h in medium and the indicated concentrations of HGF were added to serum-free medium and cultured for 15 h. Cells were pulsed with BrdU for 3 h, following which ELISA was performed according to the manufacturer's instructions. (B) TGFβ-1 inhibits proliferation. Data for both (A) and (B) were pooled from three independent experiments, $p < 0.05$.

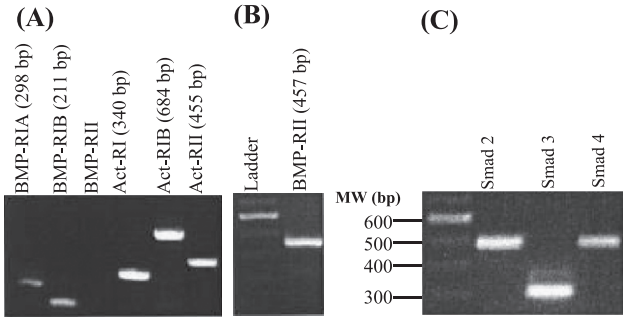

Fig. 2. (A) RT-PCR amplification of BMP and activin receptors. Molecular weights of the amplified products are provided in parentheses. Note that there is no product in the BMP-RII lane due to the use of 56 °C annealing temperature, which was higher for the primer pairs used for this gene. (B) RT-PCR amplification of BMP-RII transcript from U87 cells using 54 °C annealing temperature. (C) RT-PCR amplification of Smads 2, 3, and 4 from U87 cell RNA. All experiments were carried out using 2 μg of DNase-treated total RNA, and RT minus negative controls did not show any amplified product (data not shown).

astrocytoma cells [21,22]. Since U87 cells express TβRI and -RII, and TGFβ downregulates an important cell cycle progression protein (p27 cdk inhibitor) [16], we speculated that TGFβ inhibits the growth of these cells. Indeed, we observed that TGFβ-1 inhibits proliferation by 32% (Fig. 1B). A similar action by TGFβ has been reported in cytotoxic T cells and autologous peripheral lymphokine-activated killer cells isolated from patients with glioblastoma [31].

Given the opposing actions of TGFβ-1 and HGF on the proliferation of U87 cells, we sought to determine whether TGFβ-1 inhibits HGF secretion. Furthermore, because receptors for ligands belonging to the TGFβ family such as BMPs and activin-A are expressed in diverse regions of rodent brain [4] and are reported to play roles in astrocyte differentiation [9] and astrocytogenesis from neural progenitor cells [10,15,30], we decided to study the effects of these ligands on HGF secretion from U87 astrocytoma cells. To this end, we first assessed the expression of BMP- and activin-A receptors in U87 cells (Fig. 2A and B). RT-PCR revealed expression of all isoforms of BMP and activin receptors and Smads (2, 3, and 4) (Fig. 2C), since these proteins are the basic components of the core intracellular signaling cascade activated by the TGFβ family of receptors. To our knowledge, this is the first demonstration of expression of BMP and activin receptor isoforms in glioma cells.

These results, along with the fact that TGFβ and HGF interact at both the signaling and functional levels, led us to study the effects of not only TGFβ-1 but also BMP-2 and activin-A on HGF secretion by U87 cells. BMP-2 was chosen from among the other BMPs because it plays important roles in glial development [10,15], and it interacts with TGFβ in human glioma to antagonize TGFβ-induced inhibition of multidrug transport [31,32]. Fig. 3A shows dose-dependent inhibition of HGF secretion by both activin-A and TGFβ-1, with maximal effects at 2.5 ng/ml. BMP-2 also inhibited HGF secretion significantly, albeit to a much lesser extent than activin-A and TGFβ. Inhibition of secretion is likely transcriptional, as Northern blot analysis revealed downregulation of HGF mRNAs by TGFβ, activin-A, and BMP-2 (Fig. 4A and B). Furthermore, since HGF could induce its own receptor (c-Met) in an autocrine manner [2], we sought to determine whether inhibition of HGF by these TGFβ1 and its subfamily ligands such as activin-A and BMP-2 alters expression of c-Met in U87 cells. We observed no change in c-Met expression in response to these ligands under conditions that inhibited HGF secretion (Fig. 4C).

Reports showing differential regulation of HGF are as follows: (a) inhibition of its synthesis by cortisol and $1\alpha,25(OH)_2$-vitamin D_3 in osteoblastic cells [3,8], by angio-

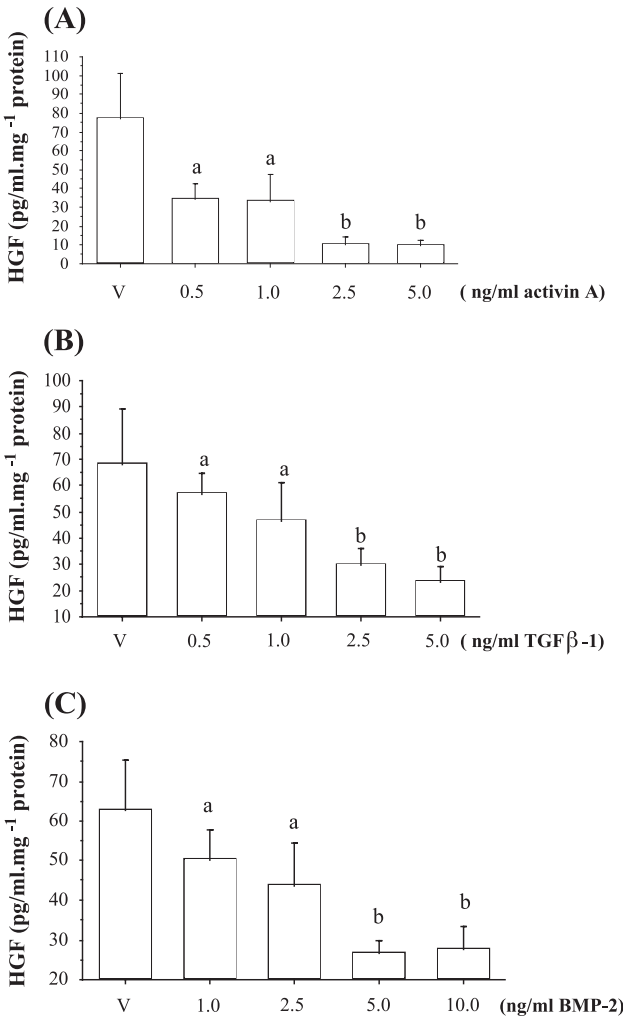

Fig. 3. U87 cells were seeded in 24-well plates and cultured in growth medium to 80% confluency. Cells were then serum-starved for 2 h, and cytokines were added at concentrations indicated. Following incubation for 18 h, conditioned medium was collected for HGF assay. Inhibition of HGF secretion by U87 cells is shown in panel A by activin-A, panel B by TGFβ, and panel C by BMP-2. Data obtained from three independent experiments ($p < 0.05$). V—vehicle; a—significantly lower than V; b—significantly lower than V and a.

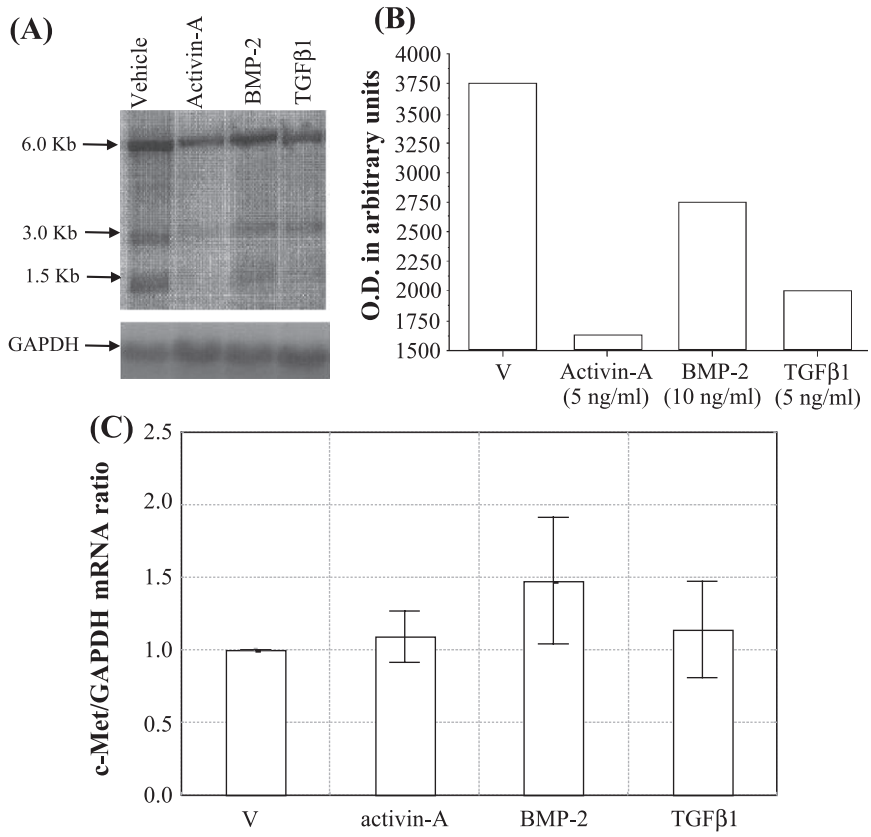

Fig. 4. (A) Northern analysis showing inhibition of all three transcripts of HGF (6.0, 3.0 and 1.5 kb) by TGFβ1, BMP-2 and activin-A compared to vehicle. (B) Densitometric analysis of all three HGF transcripts from data obtained from two independent experiments yielding similar results. (C) Quantitative determination of c-Met mRNA in U87 cells in response to TGFβ1, BMP-2 and activin-A showed no change in its expression compared to vehicle treated cells. Data pooled from three independent experiments.

tensin II and TGFβ in endothelial cells [24], and by agonists of the retinoic acid and retinoid X receptors in U87 cells [6]; (b) upregulation by activation of peroxisome proliferator-activated receptor gamma (PPARγ) in fibroblasts [18]; and (c) regulation of secretion by Raf-PKC-MEK, and a parallel p38 MAPK pathways in U87 cells [7]. Here we report downregulation of HGF by the ligands of TGFβ family members such as TGFβ-1, activin-A, and BMP-2; the first two are equipotent and the last one less potent.

Our results therefore warrant reconsideration of blocking/neutralizing TGFβR signaling as a potential therapeutic strategy, since it might obliterate TGFβ's inhibitory action on HGF secretion. Moreover, since activin-A and BMP-2 inhibits promalignant HGF, these cytokines could be considered as a way to downregulate HGF in conjunction with neutralizing/blocking TGFβ receptor signaling towards an effective combination therapy for glioma.

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