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Cell density-dependent expression of heparin-binding growth-associated molecule (HB-GAM, p18) and its down-regulation by fibroblast growth factors

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Heparin-binding growth-associated molecule (HB-GAM) is a developmentally regulated protein that is intensely expressed during the rapid postnatal growth phase of rat brain. The expression of HB-GAM studied in 12 cell lines was restricted to C6 rat glioma cell line and BALB/c 3T3 cells. In BALB/c 3T3 cells the expression of HB-GAM was enhanced in confluent and quiescent cell cultures. When the confluent cultures were treated with bFGF the expression of HB-GAM mRNA was strongly reduced and the protein disappeared rapidly from proliferating cells. The data presented suggest involvement of HB-GAM in cell differentiation phenomena rather than in cell proliferation.

Heparin-binding; Cell differentiation; BALB/c 3T3 cell; Contact inhibition; Growth factor

1. INTRODUCTION

Developing brain tissue contains many soluble growth factors and matrix-bound proteins which are involved in neuronal growth and brain maturation [1]. Recently a new family of heparin-binding molecules acting on cell growth and differentiation has been discovered. Heparin-binding growth-associated molecule (HB-GAM) [2] (also known as pleiotrophin [3] or heparin-binding neurotrophic factor [4]) was originally purified from neonatal rat brain [5] where it is expressed transiently but abundantly during the rapid axonal growth [2,5]. The expression of HB-GAM is not restricted to neuronal tissues, but low expression levels are detected transiently in other developing tissues in several species [2,3,6].

The cDNA of HB-GAM has been cloned [2,3,6] and the primary sequence, confirmed by amino acid sequencing [7,8], has revealed a secretory protein containing high amounts of basic amino acids and of cysteine. HB-GAM is structurally related to MK protein that is widely expressed in the mid-gestation period of mouse embryogenesis [9] and in embryonic stem cells which have been induced to differentiate by retinoic acid [10]. Another protein (RI-HB), which is homologous to HB-GAM and MK, has been purified from chicken embryos [11]. However, it is still an open question whether RI-HB is a third member of the protein family or the chicken analogue of MK.

HB-GAM induces neurite outgrowth of embryonic rat cerebral cells in vitro [5]. Recent studies have shown that HB-GAM is expressed and localized in developing neuronal tracts of rat brain and it could act as a neuronal guidance factor [12]. In addition to the action on neuronal differentiation HB-GAM has been suggested to have a mitogenic activity comparable to that of fibroblast growth factors [13]. However, recent data indicates that HB-GAM does not possess significant mitogenic activity [7,8,14]. Raulo et al. [14] have shown that the tissue-derived HB-GAM fractions contain contaminants which cause mitogenic effects in mouse fibroblasts.

To understand the cellular function of HB-GAM it is of great interest to define the regulation of HB-GAM expression. The aim of this study has been to find in vitro models expressing HB-GAM and to specify the possible regulatory factors of HB-GAM expression. In BALB/c 3T3 cells HB-GAM is induced by increased cell density when cells also become mitotically inactive. The expression in contact-inhibited cells can be suppressed by fibroblast growth factors at concentrations which change the quiescent phenotype to a mitotic one. The data suggest that HB-GAM is involved in the cell differentiation phenomena and suppression of cell division.

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GAPDH, gylceraldehyde-3-phosphate-dehydrogenase; HB-GAM, heparin-binding, growth-associated molecule; SDS, sodium dodecyl sulfate; TGFβ, transforming growth factor beta.

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2. EXPERIMENTAL

2.1. *Cells and cell culture*

C6 glioma cells, N18 neuroblastoma cells, SHSY-5Y neuroblastoma cells, PC12 pheochromocytoma cells, BHK cells, CHO cells, MDCK cells, 293 embryonic kidney cells, B16 melanoma cells, BALB/c 3T3 cells and WI-26 VA4 human fibroblasts were from American Tissue Culture Collection (Rockville, MD). Human primary fibroblasts (HF) were a gift from Prof. Ismo Virtanen. Recombinant acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) were from Promega (Madison, WI). Porcine transforming growth factor beta one (TGF-β1) was from R & D Systems Inc. (Minneapolis, MN).

Cells were cultured on ordinary tissue culture dishes in DMEM supplemented with 10% FCS, 100 U penicillin G/ml, and 0.1 mg streptomycin/ml in an atmosphere of 5% CO_2/95% air. In cell density assays 50% confluent BALB/c 3T3 cells were plated at a density of 2-, 2.6-, 4-, 8-, 16- and 32×10^4 cells/25 mm culture dish. The cells were cultured until the three most dense dishes become confluent. This normally took 4 days. Confluent cultures contained about 12×10^4 cells/cm^2.

2.2. *Analysis of mRNA*

Total RNA from different cell cultures was isolated using the guanidine thiocyanate method of Chomczynski and Sacchi [15]. For Northern blots total cellular RNA samples were run on 1.2% agarose–7% formaldehyde gels and stained with ethidium bromide [16]. The RNAs were transferred from the agarose–formaldehyde gels to Magna graph nylon filters (Micron Separations Inc., Westborough, MA). In Dot Blot assays 5 μg of total RNA was diluted to 250 μl of 10 mM Tris, 0.1 mM EDTA, pH 7.4, and 0.5 vol. formaldehyde, and 1 vol. 10×SSC were added (1×SSC= 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4). The samples were then denatured for 10 min at 65°C and dotted onto nylon filters by using a Bio-Dot SF microfiltration unit (Bio-Rad, Richmond, CA). RNA was covalently linked to nylon membranes with UV light.

RNA bound to the filter was hybridized with an [α-^{32}P]dCTP-labelled (Amersham, UK) HB-GAM cDNA probe [2] using QuikHyb hybridization buffer (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and exposed to Hyperfilm MP (Amersham, UK) for 16–24 h at −70°C with intensifying screens. A glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA fragment was used as a control probe [17]. Densitometry analysis of RNA from the films was carried out by using computerized image scanning.

2.3. *Antibodies and Western blotting*

Anti-synthetic peptide antibodies that bind to the N-terminal sequence of HB-GAM have been previously described [5]. For Western blotting the cultured cell samples were solubilized in phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), containing 0.1% SDS and 1 mM phenylmethyl sulfonyl fluoride at 100°C for 10 min. The samples (10 μg/lane) were run on 5–20% gradient SDS-polyacrylamide mini gels (Bio-Rad), transferred to nitrocellulose, and probed with the anti-peptide antibodies at a concentration of 0.1 μg/ml, as described in detail elsewhere [5]. Antibodies bound to protein bands were located using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and the light-based detection system, ECL (Amersham, UK).

3. RESULTS

3.1. *Expression of HB-GAM in cultured cell lines*

The expression of HB-GAM mRNA in twelve cell lines was studied by Northern blot hybridization analysis (Fig. 1). In the cell lines studied a strong HB-GAM expression was observed in C6 rat glioma cells and BALB/c 3T3 fibroblasts, which gave an intense band of 1.9 kb in Northern blotting. Only very faint or no detection was found in the other cell lines studied (Fig. 1).

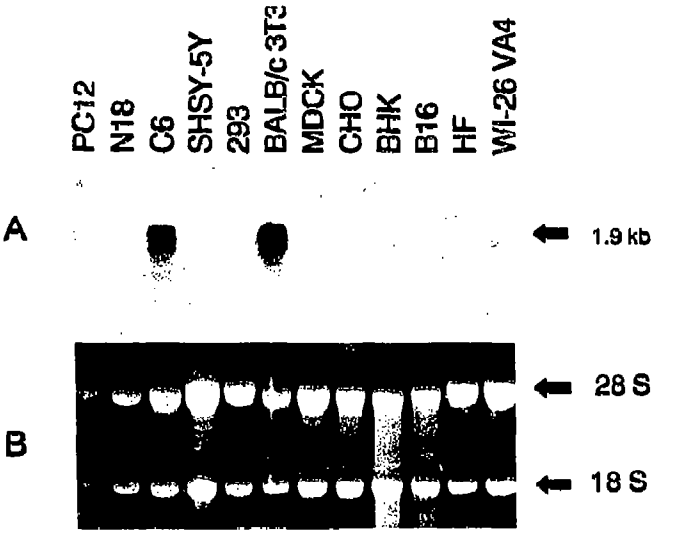

Fig. 1. Expression of HB-GAM in different cell lines. (A) Cells were grown and total RNA was extracted and hybridized with the HB-GAM probe as described in section 2. 15 μg of total RNA was loaded to each lane. (B) Ethidium bromide staining of the RNA gel.

3.2. *Dependency of HB-GAM expression on cell density*

In BALB/c 3T3 cells the expression of HB-GAM mRNA varied in different experiments. Cells growing sparsely in culture expressed low levels of HB-GAM, but when cells were allowed grow to confluency the relative expression of the HB-GAM mRNA was greatly increased (Fig. 2) Densitometry analysis revealed a more than 6-fold higher expression in confluent as compared to sparse cultures (Fig. 2). In contrast, no change or a slight decrease was observed in GAPDH mRNA (Fig. 2). In C6 glioma cells a similar trend was observed but the difference in mRNA expression between sparse and dense cell cultures was smaller (not shown).

3.3. *Effect of bFGF on HB-GAM expression*

A well-known feature of BALB/c 3T3 cells is the fact that growth factors, like bFGF and aFGF, can turn confluent, quiescent BALB/c 3T3 cultures mitotic and cause them to lose their contact-dependent inhibition of cell division. To study whether the cell density-dependent high expression of HB-GAM can be reversed with growth factors, the cultures were treated with bFGF at concentrations normally used in assays of mitogenic activity. In 36-h experiments the HB-GAM mRNA expression was reduced to less than 2% when measured with densitometry scanning (Fig. 3A). The reduction of the expression was observed at all concentrations higher than 5 ng/ml (Fig. 4A). These bFGF concentrations also caused the cells to lose their contact-inhibited phenotype. The HB-GAM mRNA levels started to decrease rapidly after addition of bFGF (Fig. 4B). After 24 h

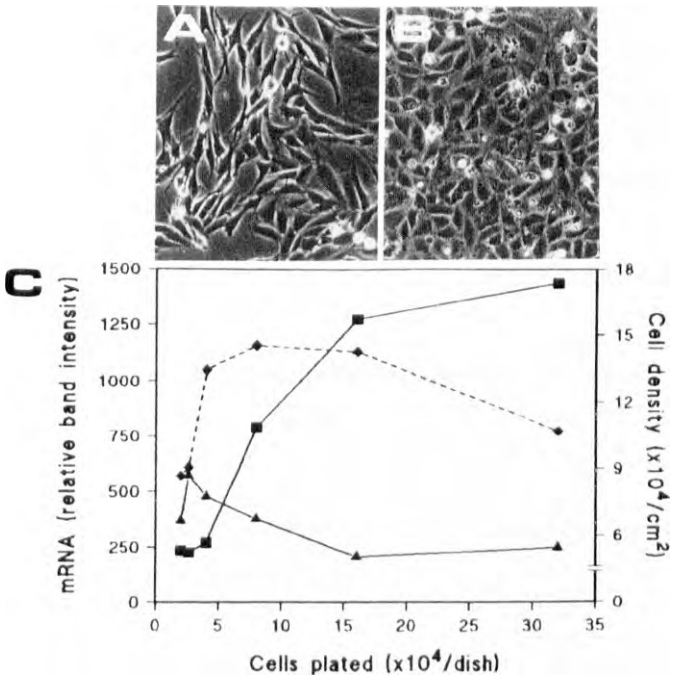

Fig. 2. Effect of cell density on HB-GAM expression. Phase contrast micrographs show the morphology of sparse (A) and of confluent (B) cultures. (C) The relationship between the density (◆–◆) of BALB/c 3T3 cells and HB-GAM (■–■) or GAPDH (▲–▲) expression. 5 μg of RNA from cultures at different cell densities were hybridized with the cDNA probes (see section 2). Autoradiography film was analyzed with computerized image scanning.

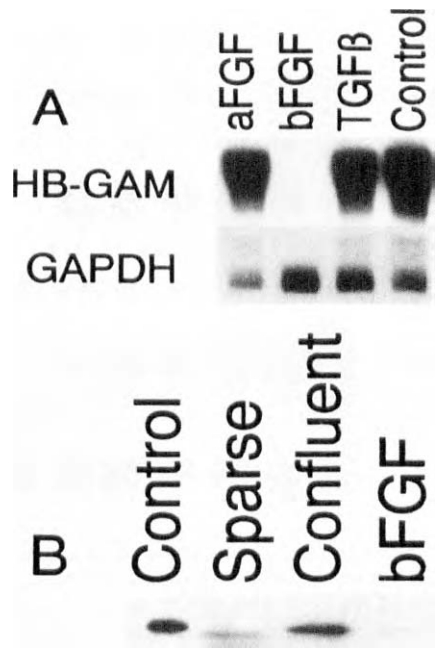

Fig. 3. Effect of growth factors on HB-GAM expression. (A) BALB/c 3T3 cells were cultured to confluency and then treated with 10 ng/ml aFGF, bFGF or TGFβ_1 for 36 h. RNA was extracted and hybridized with the cDNA probes. (B) Western blotting of HB-GAM from sparse, confluent and confluent bFGF-treated BALB/c 3T3 cells with anti-synthetic peptide antibodies that bind to the N-terminal sequence of HB-GAM. 10 μg of protein from cultured cells was loaded to each lane. The control band corresponds to 50 ng of recombinant HB-GAM.

94% of the HB-GAM mRNA had disappeared from the cells.

aFGF was unable to reduce HB-GAM expression when used at the same concentration as bFGF (Fig. 3A). However, when the aFGF concentration was raised to 100 ng/ml the HB-GAM mRNA expression was reduced as with bFGF (not shown). TGFβ_1 did not change the HB-GAM level at 10 ng/ml (Fig. 3A).

The regulation of HB-GAM expression in BALB/c 3T3 cells was confirmed by immunoblotting analysis (Fig. 3B). When cell lysates from sparse and confluent cultures were studied by Western blotting a distinct increase in HB-GAM expression was observed in dense 3T3 cell cultures (Fig. 3B). In lysates from confluent BALB/c 3T3 cultures treated with bFGF for 24 h and analyzed by Western blot experiments the immunoreactive 18 kDa band of HB-GAM was greatly reduced compared to confluent cell cultures without bFGF (Fig. 3B).

4. DISCUSSION

Several lines of evidence suggest that HB-GAM plays an important role in brain maturation. Both the tissue-derived and purified recombinant HB-GAM clearly induce axonal growth in brain neurons in vitro [5,8,14]. The expression of HB-GAM in rat brain tissue takes place simultaneously with rapid axonal growth and network formation. Recent studies also suggest that HB-GAM is localized to developing neuronal tracts and mediates axonal guidance in vitro [12]. The possible mitogenic properties of HB-GAM have been studied in several laboratories giving controversial results [7,8,13,14], and any conclusions confirming mitogenic function cannot be drawn.

The purpose of this study has been to investigate regulative mechanisms of HB-GAM expression. The expression of HB-GAM is restricted among different cell lines suggesting distinct regulation of the HB-GAM gene. HB-GAM expression is enhanced in BALB/c 3T3 cells upon contact inhibition of cell proliferation. Conditioned medium collected from confluent cells did not change HB-GAM expression in sparsely cultured BALB/c 3T3 cells (Merenmies, unpublished results) suggesting that HB-GAM expression is independent of soluble factors and might be caused by cell-to-cell contacts. The expression is increased after the cells have reached confluency, which suggests that HB-GAM is not the reason for contact inhibition but rather the consequence. This also appears to be the case during rat brain development when HB-GAM expression increases rapidly once the mitotic activity of brain neurons has ceased [2,5].

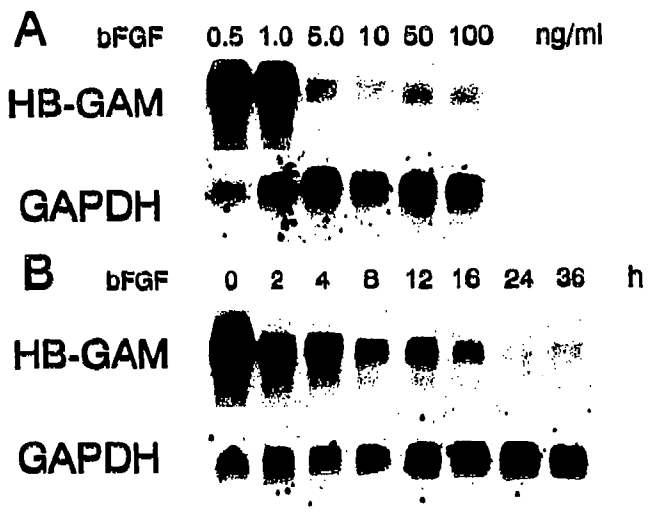

Fig. 4. Dose–response (A) and time–course (B) for bFGF suppression of HB-GAM mRNA expression in BALB/c 3T3 cells. (A) Confluent cells were incubated for 24 h with bFGF at the concentrations indicated. (B) Confluent cells were cultured with 10 ng/ml of bFGF for indicated time periods. RNA was extracted and hybridized with the cDNA probes.

The enhancement of HB-GAM expression is reversed by bFGF, and also with aFGF at higher concentrations, which cause mitotic activation of the cells and loss of the contact-inhibited cell shape. The reduction in HB-GAM mRNA is rapidly reflected to the HB-GAM protein levels, suggesting that HB-GAM has a short half-life in actively dividing cells. This might be due to enhanced proteolytic mechanisms like the plasminogen-plasmin system, which is known to be activated by bFGF [18]. Retinoic acid treatment of C6 glioma cells and BALB/c 3T3 cells did not change the HB-GAM expression (Merenmies, unpublished results). This agrees with previous studies [19], and suggests that HB-GAM is regulated differently from the MK and RI-HB proteins that belong to the same protein family [10,11].

Induction of HB-GAM expression in vitro by saturating cell density and the ability of bFGF to reverse this cell density-dependent induction do not support the idea of a mitogenic role but are consistent with the view that HB-GAM is involved in the cell differentiation phenomena.

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