Autocrine factors sustain glioblastoma stem cell self-renewal

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Abstract. Glioblastoma stem cells are able to reform original glioblastoma and express the neural stem cell marker CD133 and Nestin. They can self-renew and proliferate in tumor sphere medium containing EGF, bFGF and LIF that is known to be permissive for stem cell proliferation. In this study, we found that neurosphere-like colonies appeared after the human primary glioblastoma cells had been switched into pure DMEM/F12 medium. We investigated whether tumor spheres formed in pure DMEM/F12 medium possess the characteristics of glioblastoma stem cells. We identified that the tumor sphere cells were cancer stem cells of glioblastoma and they can self-renew and proliferate in pure DMEM/F12 medium. Glioblastoma cells can secrete several factors that result in autocrine motility signaling and stimulate glioma invasion. We hypothesized that an essential autocrine signal promotes the self-renewal and proliferation of human glioblastoma stem cells in pure DMEM/F12 medium. Then, expression of EGF and bFGF in glioblastoma stem cells were analyzed. Both the mRNA and protein of EGF and bFGF were detected in three human glioblastoma stem cells. Our findings suggest that autocrine of EGF and bFGF may sustain the self-renewal of glioblastoma stem cells.

Introduction

Cancer stem cells (CSCs) are widely thought to be able to initiate and reform the original cancer after CSCs were isolated and identified from more and more solid tumor and leukemia (1-5). They share many characteristics of embryonic stem cells and normal tissue stem cells. They possess the property of multipotential differentiation, unlimited proliferation and

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self-renewal (6,7). The capacity of self-renewal plays a key role in retaining stem cell-like properties and maintaining CSC pool.

Brain tumor stem cells (BTSC) isolated from human glioblastoma (GBM) express the cell surface marker CD133, (3,8,9) a neural stem cell marker. They are able to reform original glioblastoma and divide, amplify both undifferentiated and differentiated cancer cells after *in vivo* implantation. We have isolated BTSC from human primary glioblastoma tissue and identified its characteristics of CSCs (10). We found that a few glioblastoma cells were able to form neurosphere-like colonies while the primary human glioblastoma cells were switched into pure DMEM/F12 medium (without fetal bovine serum and exogenous growth factors). Then, the biological characteristics of this subpopulation of glioblastoma cells were analyzed. We demonstrated that the subpopulation of glioblastoma cells were BTSCs. The subpopulation also showed self-renewal in pure DMEM/F12 medium without fetal bovine serum (FBS) and exogenous growth factors. We show that there were expression of EGF and bFGF in BTSCs. Our findings suggest that autocrine of EGF and bFGF may sustain glioblastoma stem cell self-renewal.

Materials and methods

Culture of primary GBM cells and tumor sphere. Three human GBM samples were obtained from consenting patients, as approved by the Research Ethics Boards at The Xinqiao Hospital. Tumors were washed, acutely dissociated in DMEM/F12 medium without serum and subject to enzymatic dissociated. Tumor cells were then resuspended in DMEM/F12 medium containing 10% fetal bovine serum (FBS) as growth medium and plated at a density of $2x10^6$ live cells per 75 cm^2 flask. The cells attached and grew as a monolayer in flasks. The primary glioblastoma cells were harvested, dissociated and washed by pure DMEM/F12 medium three times. Then, all the three GBM cells were cultured in pure DMEM/F12 medium.

Subsphere forming assay. After primary spheres formed and reached 50-100 cells each in the culture, sphere cells were harvested, dissociated, washed by pure DMEM/F12 medium three times and plated in 96-well microwell plates for the subsphere-forming assay by limiting dilution, as described previously (3,11). In brief, the cells in single-cell suspension were diluted and plated at 100 cells/well. Cells were fed with

0.2 ml volumes of serum-free DMEM/F12 medium. Cultures were fed 0.025 ml of fresh pure DMEM/F12 medium every 2 days. The wells were scored for sphere formation after 14 days.

Cell proliferation assay. The subsphere cells were harvested, dissociated into single cells, washed by pure DMEM/F12 medium three times and plated at 1000 cells/well into a 96-well plate in 0.2 ml volumes of pure DMEM/F12 medium. Half of the medium in every well was changed every 2 days. Cell proliferation assays were performed on days 1, 3, 5, 7 and 9 postplating using the Dojindo WST-8 [2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]-based cell counting kit-8 (Dojindo Biotechnology, Japan). Quantification of viable cells through reading of UV absorption spectrums at 450 nm was performed on a microplate reader.

Immunocytochemical staining of GBM sphere cells. For immunostaining of undifferentiated tumor spheres, spheres were plated onto poly-L-lysine coated glass coverslips in pure DMEM/F12 medium for 4 h. Cells were then fixed with 4% paraformaldehyde and stained with antibodies against CD133 (goat polyclonal IgG; Santa Cruz Biotechnology), Nestin (mouse monoclonal IgG; Santa Cruz Biotechnology), glial fibrillary acidic protein (GFAP, mouse monoclonal IgG; Santa Cruz Biotechnology), myelin basic protein (MBP, rabbit polyclonal IgG; Santa Cruz Biotechnology), mitogen-activated protein 2 (MAP2, mouse monoclonal IgG; Santa Cruz Biotechnology). Appropriate SP Cytostain-Plus kits (Zhongshan Biotechnology) were used. Color visualization was performed using 3-3'-diaminobenzidine as the chromagen substrate (Zhongshan Biotechnology).

The glioblastoma sphere cells were dissociated into single cells. CD133-2-phycoerythrin (10 μl) (fluorochrome-conjugated mouse monoclonal IgG1; Miltenyi Biotec) was added for 30 min and the percentage of CD133+ cells were counted by flow cytometry. The experiment was repeated three times.

Implantation into nude mice. Five Balb/C nude mice (6 weeks old; The Experimental Animal Laboratories, Shanghai, P.R. China) were anesthetized with i.p. ketamine and xylazine and then were treated with VP-16 (etoposide), 30 mg/kg, diluted in serum-free HBSS, via an i.p. injection. Seven days later, the mice were anesthetized as described above and the regions of the upper extremity were implanted subcutaneously with both isolated GBM sphere cells (right, 10000 cells per mouse) and primary GBM cells (left, 1000 000 cells per mouse) in 0.2 ml 0.01 M PBS. Tumor growth was monitored weekly. The mice were sacrificed after 6 weeks of the implantion. The same experiment was repeated once with identical situations. All the animal experiments were in strict accordance with the Institutional Animal Care guidelines.

Immunohistochemistry on tumor sections. Formalin-fixed, paraffin-embedded tissue sections were mounted on positive-charged microscope slides. Tissue sections were then baked overnight at 60˚C and treated with epitope retrieval techniques and blocked for endogenous peroxidase and biotin before the application of the primary antibody. Incubation of antihuman GFAP at a 1:100 dilution was performed overnight at 4˚C. Subsequent immunodetection was performed using SP Histostain-Plus kits (Zhongshan Biotechnology). Color visualization was performed using 3-3'-diaminobenzidine as the chromagen substrate (Zhongshan Biotechnology).

RNA extraction and RT-PCR. Total RNA of glioblastoma subsphere cells was extracted by Tripure Reagent (Roche, USA). Retrotranscription was carried out with RNA for each sample using RT M-MLV (Toyobo, Japan) in the presence of random primers. The PCR was carried out in a 20 μl reaction mixture that contained 1 μl cDNA as template, specific oligonucleotide primer pairs, and Accuzyme (Toyobo). EGF (forward GGATAGCCAACAAACACACT, reverse GGCACGTGCAGTAATAGGAT): 36 cycles of 30 sec at 94˚C, 30 sec at 60˚C and 90 sec at 72˚C; bFGF (forward CCTGGGGAGAAAGCTAT, reverse GCTTCACGGGTAA CAG): 36 cycles of 30 sec at 94˚C, 30 sec at 58˚C and 90 sec at 72˚C. Sense and antisense primers were synthesised according to the sequences extracted from GenBank. The amplified products were identified by agarose gel electrophoresis and ethidium bromide staining.

Immunoblotting. Cell extracts were prepared with RIPA kit (KeyGen Tec, Nanjing, P.R. China) and resolved on a 10% SDS-PAGE and blotted onto a membrane. After blocking at 37˚C in 5% milk in PBS containing 0.1% Tween-20 for 2 h, membranes were incubated overnight with antibodies against EGF, bFGF or ß-actin proteins (Santa Cruz Biotechnology). After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated appropriate secondary antibodies followed by standard chemiluminescent substrate (Pierce Biotechnology).

Results

Primary GBM form neurosphere-like colonies in pure DMEM/F12 medium. Studies on BTSCs have showed that BTSCs is able to proliferate and form neurosphere-like colonies cultured in serum-free tumor sphere medium (TSM) containing EGF, bFGF and LIF (3,8,12). When primary GBM cells were switched into pure DMEM/F12 medium, neurosphere-like colonies appeared in all three GBM and reached ~50 cells each sphere in 2 weeks (Fig. 1A). It was smaller than the tumor spheres cultured in TSM. The tumor spheres generated from 3 specimens remained nonaderent and exhibited immunoreactivity for CD133 and Nestin. A few cells within tumor spheres were also found to be positive for lineage markers MBP (for oligodendrocytes), but no GFAP (for astrocytes) and MAP2 (for neurons) staining was detected within spheres (Fig. 1B-F). The cells were stained with antibody conjugated PE against CD133. The frequency of CD133+ cell population within the primary tumor spheres was determined by flow cytometric assays, yielding CD133+ cell frequency to 68.8% (Fig. 2).

GBM sphere cells exhibit potential for self-renewal and can proliferate in pure DMEM/F12 medium. Tumor spheres are defined as clonally derived nonadherent colonies of cells
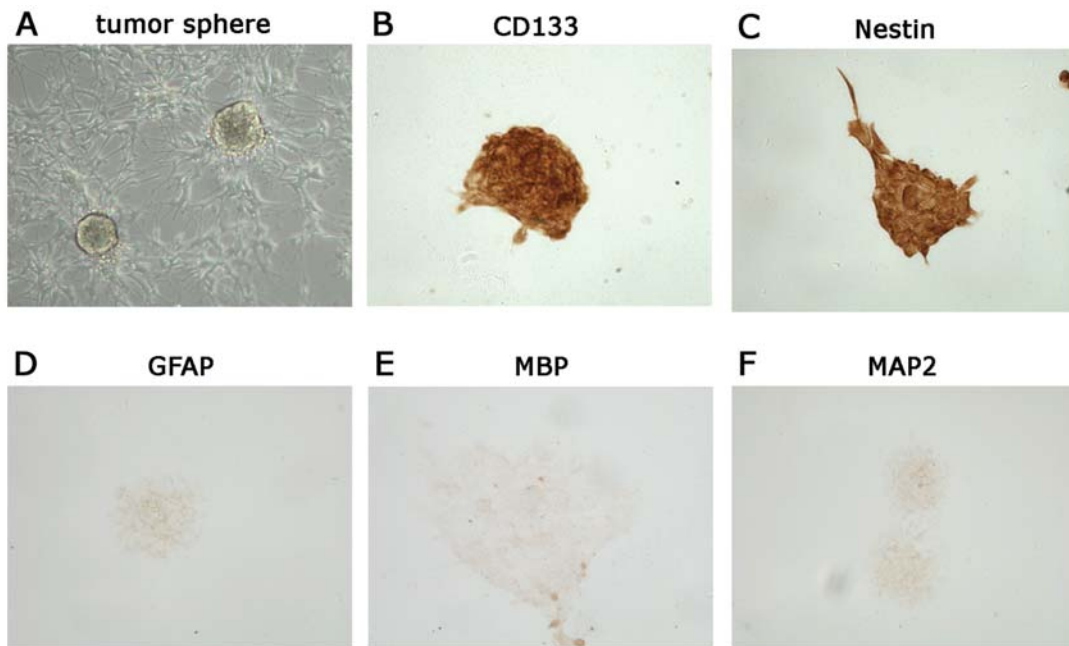
Figure 1. Neurosphere-like colonies appeared in GBM cells cultured in pure DMEM/F12 medium (A, magnification x100). Glioblastoma spheres were stained by NSC markers CD133 (B, magnification x400) and Nestin (C, magnification x400). Lineage markers for oligodendrocyte, MBP (E, magnification x400), were stained, but staining for the astrocyte marker, GFAP (D, magnification x400), and neuron, MAP2 (F, magnification x400), was not observed in the glioblastoma sphere.
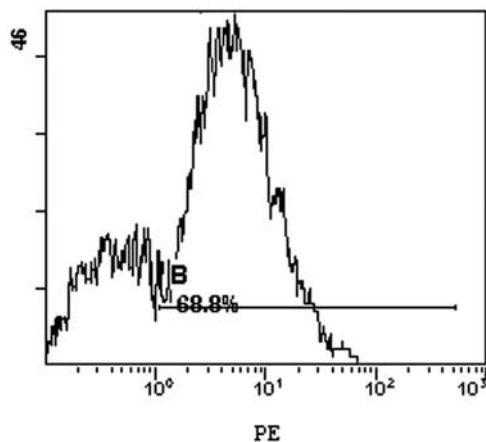

Figure 2. Flow cytometry histogram in representative glioblastoma sphere cells, with the first peak (left) representing cells negative for CD133-phycoerythrin expression and the second peak (right, gate B) representing CD133-positive cells.

derived from a single tumor stem cell. We analyzed the ability of the primary GBM sphere cells to continue proliferating and forming secondary sphere with a sphere-forming assay. After disassociation and replating at ~100 cells/well, all of the dissociated primary tumor spheres demonstrated the capacity to form secondary tumor spheres, exhibiting ability to self-renew in pure DMEM/F12 medium. It generated 5.04±2.39 secondary tumor spheres every 100 primary sphere cells. Morphology of secondary tumor spheres was identical to that of primary spheres and secondary tumor spheres retain expression of the neural stem cell markers CD133 and Nestin (Fig. 3). These data show that the CSCs of human glioblastoma had the capacity to self-renewal in pure DMEM/F12 medium.

The primary sphere cells were plated at 1000 cells/well cultured in pure DMEM/F12 medium and the number of viable cells was quantified on days 1, 3, 5, 7 and 9 after plating by the WST-8 colorimetric assay. It was demonstrated that the primary glioblastoma sphere cells were able to proliferate when they were cultured in pure DMEM/F12 medium. But its capacity of proliferation in pure DMEM/F12 medium was significantly lower than BTSCs cultured in TSM (Fig. 4). These data show that the primary glioblastoma spheres formed in pure DMEM/F12 medium had the capacity for self-renew and proliferation.

Glioblastoma spheres cultured in pure DMEM/F12 medium are capable of forming tumors in vivo. To determine whether the glioblastoma spheres are capable of tumor initiation *in vivo*, we implanted the isolated glioblastoma spheres cultured in pure DMEM/F12 medium into the upper extremity of nude mice subcutaneously. There was no tumor detected 6 weeks after the implantation of 1000,000 primary glioblastoma cells. Three mice developed tumor after 10,000 tumor sphere cells implantation in 5 mice. The tumor cells, which developed in mice after subcutaneous implantation of human glioblastoma spheres, stained positive for anti-GFAP antibody (Fig. 5). These results suggested that the glioblastoma spheres cultured in pure DMEM/F12 medium had the capacity to initiate tumor *in vivo* and possess characteristics of BTSC.

Glioblastoma sphere cells can autocrine EGF and bFGF. Astrocytes, oligodendrocytes and microglia can express EGF, hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α) and others (13-15). Glioblastoma cells also secrete several factors that result in autocrine motility signaling and stimulate glioma invasion (15-17). The
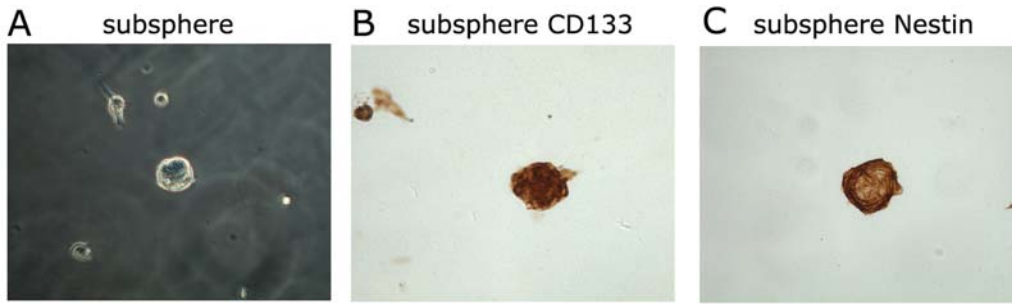
Figure 3. Tumor sphere cells exhibit self-renewal potential. Morphology of secondary tumor spheres (magnification x200) was identical to that of primary tumor spheres (A). Undifferentiated secondary tumor spheres (magnification x400) retain expression of neural stem cell markers CD133 (B) and Nestin (C).
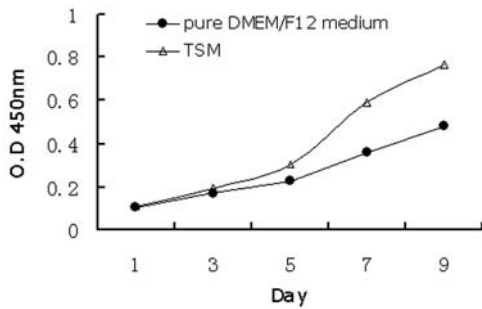

Figure 4. Tumor sphere cells exhibit proliferation cultured in pure DMEM/F12 medium. Tumor sphere cells cultured in pure DMEM/F12 medium showed a lower degree of cell proliferation than cells cultured in TSM.

epidermal growth factor receptor (EGFR) gene is commonly amplified and rearranged in glioblastoma multiforme. Expression of wild-type EGFR ligands, such as transforming growth factor-α (TGF-α) or heparin-binding EGF (HB-EGF), is also often increased in gliomas resulting in an autocrine loop that contributes to the growth autonomy of glioma cells (15,16). Therefore, we hypothesized that an essential autocrine signal promotes the self-renewal and proliferation of human glioblastoma stem cells in pure DMEM/F12 medium. To better define the autocrine of glioblastoma stem cells, the total RNA and protein of glioblastoma subsphere cells were extracted. Expression of EGF and bFGF was assessed by RT-PCR and Western blot assay. As shown in Fig. 6 EGF and bFGF mRNA were amplified in all three glioblastoma subspheres. The protein of EGF and bFGF also were detected in three glioblastoma stem cells. These results are consistent with the above hypothesize on autocrine of glioblastoma stem cells.
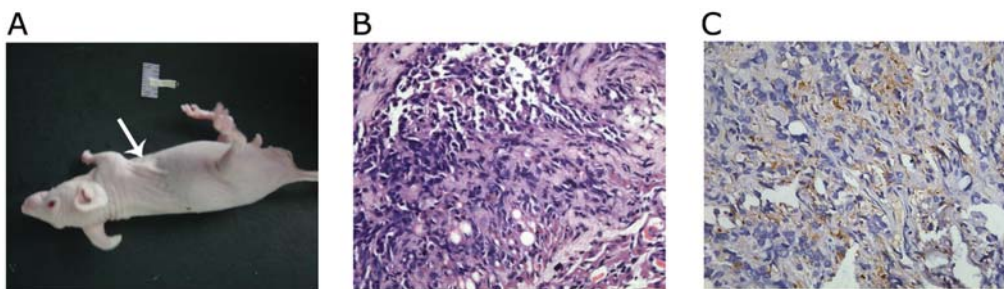

Figure 5. Evidence of *in vivo* tumor-forming ability of glioblastoma spheres. The isolated glioblastoma spheres were able to form tumors in nude mice after subcutaneous implantations (A), but the primary glioblastoma cells did not, even when implanted cell number reached 100 times that of the glioblastoma sphere cells. The tumors developed in the mice were stained by hematoxylin and eosin and could be stained by human-specific antibodies against GFAP.
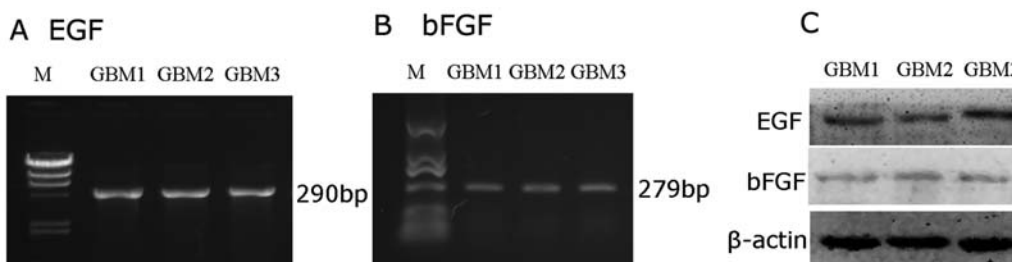

Figure 6. Semiquantitative RT-PCR and Western blot analysis of expression of EGF and bFGF in glioblastoma stem cells. Semiquantitative RT-PCR showed mRNA transcription levels of EGF (A) and bFGF (B) in three cases of glioblastoma stem cells. Western blot analysis confirmed levels of EGF and bFGF protein in three cases of glioblastoma stem cells (C).

Discussion

The concept of CSC arose from the striking similarities between the self-renewal mechanisms of stem cell and a small fraction of cancer cells in tumors (7). One key determinant of stem cells and CSCs is the capacity for self-renewing and extensive proliferation (18,19). BTSCs were isolated with a remarkable potential for self-renewal and these cells were found to drive the formation and growth of tumors (3,8,12). Recent studies found that CSCs of human GBM can self-renew and proliferate in TSM (3,8). TSM known to be permissive for stem cell proliferation was established previously for isolation of neural stem cell as neurospheres (3,8,20). Therefore, TSM become a common culture medium of human BTSCs.

Recently, we have isolated CSCs of human glioblastoma and identified the capacity of self-renewing, proliferation and tumor initiation. When we switched the human GBM primary culture cells into TSM containing EGF, bFGF and LIF, we also switched it into pure DMEM/F12 medium without exogenous growth factors or FBS. Neurosphere-like colonies appeared in all three GBM cultures after 1 week and reached ~50 cells each after 2 weeks in pure DMEM/F12 medium. This raises the question: do the tumor spheres consist of BTSCs? To answer this question, the sphere cells were subjected to a self-renewing, proliferation and initiating tumor assay.

In this study, we have identified that the tumor sphere cells cultured in pure DMEM/F12 medium consist of BTSCs. There are several lines of evidence that support this: a) the tumor sphere cells showed immunoreactivity for CD133, a neural stem cell marker and BTSC marker; (21-25) b) the tumor sphere cells can self-renew and proliferate to generate subspheres that express CD133; c) the isolated tumor sphere cells can produce brain tumors in nude mice, its capacity of initiating tumor is much stronger than the cultured primary glioblastoma cells. Then, it is intriguing to speculate why BTSCs could self-renew and proliferate in pure DMEM/F12 medium. Are the self-renewal and proliferation of BTSCs independent of exogenous growth factors?

The serum-free medium (SFM) of culturing BTSCs used previously contained bFGF, EGF, LIF and/or PDGF. SFM allows for the maintenance of an undifferentiated stem cell state, and the addition of bFGF and EGF in TSM induced the proliferation of multipotent, self-renewing, and expandable neural stem cells (11,26,27). Our data show that the BTSCs of human glioblastoma could self-renew and proliferate in pure DMEM/F12 medium without exogenous growth factors. What is the mechanism? Previously, GBM cells were shown to expressed EGFR, FGFR and PDGFR and they could secrete several autocrine factors such as EGF, bFGF, PDGF and promoted invasion (15,17,28-30). The autocrine factors can promote invasion of GBM cells and also can promote self-renewing and proliferation of BTSCs. Results of RT-PCR and Western blot analysis show that there were expression of EGF and bFGF in three human GBM BTSCs. The data suggest that the autocrine factor may sustain BTSCs of human GBM self-renewal and proliferation.

In this study, we provide new insight into the self-renewal and proliferation of human BTSCs. With evidence of self-renewal, proliferation and initiating tumor, we identified that BTSCs can self-renew and proliferate in serum-free medium without exogenous growth factors. We also provide evidence to support that there were autocrine factors EGF and bFGF in human BTSCs. The finding of self-renewal in serum-free medium without exogenous growth factors and autocrine factor expression in BTSCs has important implications for understanding the molecular mechanisms of self-renewal and proliferation in human GBM BTSCs.

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