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Mini-review

The role of basic fibroblast growth factor in glioblastoma multiforme and glioblastoma stem cells and in their *in vitro* culture

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ABSTRACT

Glioblastoma multiforme (GBM) is the most malignant form of central nervous system tumor, and current therapies are largely ineffective at treating the cancer. Developing a more complete understanding of the mechanisms controlling the tumor is important in order to explore new possible treatment options. It is speculated that the presence of glioblastoma stem or stem-like cells (GSCs), a rare type of pluripotent cancer cell that possesses the ability to self-renew and generate tumors, could be an important factor contributing to the resistance to treatment and deadliness of the cancer. A comprehensive knowledge of the mechanisms controlling the expression and properties of GSCs is currently lacking, and one promising area for further exploration is in the influence of basic fibroblast growth factor (FGF-2) on GSCs. Recent studies reveal that FGF-2 plays a significant part in regulating GBM, and the growth factor is commonly included as a supplement in media used to culture GSCs *in vitro*. However, the particular role that FGF-2 plays in GSCs has not been as extensively explored. Therefore, understanding how FGF-2 is involved in GSCs and in GBMs could be an important step towards a more complete comprehension of the managing the disease. In this review, we look at the structure, signaling pathways, and specific role of FGF-2 in GBM and GSCs. In addition, we explore the use of FGF-2 in cell culture and using its synthetic analogs as a potential alternative to the growth factor in culture medium.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and most malignant type of central nervous system (CNS) tumor with a median survival rate of less than two years [1]. Current treatments (usually surgical resection followed by radiation or chemotherapy) generally fail to control progression of the tumor, and recurrence is practically inevitable [2]. It is speculated that this is partially due to the presence of a heterogeneous cell population within the tumor, with glioblastoma stem cells (GSCs) at the top of the hierarchy.

In recent years, there has been a growing appreciation of the connection between oncology and stem cell biology [3]. The theory of cancer stem cells (CSCs) stands at the nexus of these two fields in its postulation of the existence of a specialized subset of tumor cells that are stem cell-like [4]. Following the hierarchical model of stem cells, CSCs are uniquely endowed with the ability to recapitulate the original tumors in xenografts. They are thought to cause tumor initiation and are also known as tumor-initiating cells. They are largely resistant to the most advanced and rigorous modern chemo- and radiation therapies and are thus hypothesized to be the culprit behind the frequent tumor relapse in patients. The first experimental evidence for their existence and their characteristics was demonstrated in acute myeloid leukemia in the seminal work by Bonnet and Dick [5]. Similar evidence has emerged for solid tumors, including breast [6], pancreatic [7,8], colon [9,10], and brain tumors [11,12].

The existence of GSCs was first demonstrated by Singh et al. [11,12]. Galli et al. similarly demonstrated that glioblastoma cell lines possess molecular, cytologic, and histologic characteristics similar to neural stem cells (NSCs) [13]. Like other CSCs, GSCs are especially endowed with the ability to resist radiation therapy [14–17]. For example, GSCs were shown to possess higher capacity to activate DNA damage checkpoint proteins and thereby are more radiation resistant than non-GSCs [14]. To overcome GSC radiation resistance, DNA damage checkpoint activation [14], Notch signaling [18], HSP90 activity [19], and Wnt/β-catenin signaling [20] have been suggested as possible targets. Many of these pathways are important in non-tumorigenic normal stem cells [4] and thus further highlight the connection between stem cell biology and oncology.

Generally, the role of receptor tyrosine kinases (RTKs) in GBM has been well-documented. The most extensively studied RTK in

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GBM is the epidermal growth factor receptor (EGFR). Either an amplification or variant spliced form of EGFR (i.e. EGFRvIII) is found in more than half of the patients and has been linked with aggressive subtypes of GBM [21,22]. The roles of additional RTKs, on the other hand, have not been as extensively studied. A growing recognition of their importance has developed, since, for example, RTKs such as the platelet derived growth factor α (PDGFRA), hepatocyte growth factor receptor (HGFR/MET), and fibroblast growth factor receptor (FGFR) were also found to be frequently altered or amplified [23]. Increasing evidence demonstrates the roles and importance of FGFs in GBM; however, the role of FGFR in glioblastoma stem cell (GSC) biology is less well understood. A better insight into the FGFR/fibroblast growth factor (FGF) axis provides an opportunity to improve our overall comprehension of GSCs.

This review will therefore primarily focus on the role of basic FGF (bFGF/FGF-2) in GBM and GSCs. Emerging evidence demonstrates the critical role of FGF signaling in GBM and GBM stem cell-like cell lines [24,25]. It is also important to mention that FGF-2, along with epidermal growth factor (EGF), is an essential component of *in vitro* culture media for NSCs and GSCs [12,26]. We will explore the current understanding of FGF-2, its signaling pathways, its downstream effects, and its use in GSC culture.

2. Structure and isoforms of basic fibroblast growth factor

The FGF superfamily is made up of 22 different fibroblast growth factor genes [27]. Basic FGF (FGF-2) and acidic FGF (FGF-1) are unique in that they do not follow the conventional signal sequence for secretion [28]. Furthermore, FGF-2 is found in several isoforms. Five known isoforms of FGF-2 exist via alternative initiation of translation. The 18 kDa low molecular weight (lmw) form follows the classic Kozak initiation AUG codon. Four upstream CUG start codons generate high molecular weight (hmw) forms of 22, 22.5, 24, and 34 kDa FGF-2. While the lmw form can be secreted, the high molecular weight isoforms cannot. They remain in either the cytosol or the nucleus. The secreted FGF-2 can be internalized by target cells and are translocated into their cytoplasm and nucleus [29]. Further details on the role of different isoforms can be found in an excellent review by Sorensen et al. [28]. More specifically to our discussion here, it was found that the nuclear accumulation of hmw FGF-2 is associated with glioma cell proliferation [30]. The lmw FGF-2 was also reported to have a similar effect once internalized, by being regulated by FGF-2 interacting translokin and gaining a C-terminal nuclear localization sequence for specific targeting to the nucleus [31]. It is still unclear how each of the FGF-2 isoforms correlates with stem cell phenotype maintenance and remains to be elucidated. If a specific isoform can be linked to GSC enrichment, it has interesting potential to generate GSC-specific analogs (see Section 5) or a more optimal media development for their growth *in vitro*.

3. Basic fibroblast growth factor signaling pathways in glioblastoma and glioblastoma stem cells

FGF-2 can undergo several alternate signaling pathways depending on the isoform, localization, and cell conditions. Endogenous lmw FGF-2 can be released from the cell and can signal either in autocrine or in paracrine manner. It lacks a definitive secretion signal sequence and is released by an ER/Golgi-independent mechanism that relies on its association with other molecules [32]. Lmw FGF-2 signals through the fibroblast growth factor receptors (FGFRs) and binds primarily to FGFR-1 and FGFR-2 [32]. Lmw FGF-2 first binds to heparin sulfate proteoglycans (HSPGs), and this complex then binds to the FGFR, inducing a signal transduction pathway [33]. The formation of the primary FGF-2/HSPG complex is necessary to stabilize FGFR dimerization [34]. Several different signaling pathways can be activated depending on the downstream formation of multi-docking signaling complexes via tyrosine phosphorylation [32]. The docking protein FRS2 is responsible for recruiting a majority of proteins involved in FGF-2 signaling pathways. A total of six Grb2 molecules are recruited either directly by FRS2 or indirectly by a FRS2/Shp2 complex. Grb2 molecules then recruit either SOS, which leads to activation of the Ras-MAKP pathway, or Gab1, which leads to activation of the PI3K-Akt pathway [33], which is associated with proliferation [35], angiogenesis [36], and survival [37].

FGF-2 is known to modulate the apoptosis pathways [38] and thereby promotes survival via resistance to radiation induced cell death [39]. For carcinoma stem cells defined as Hoechst dye effluxing side population (SP) cells, the FGF-2 pathway was found to affect DNA repair [40]. In addition, the SP was found to have a highly constitutive active expression of FGF-2, again signifying the importance of FGF-2 in CSCs.

In a recent study looking at GBM, it was found that secretion of FGF-2 by GBM cells enhances the blood brain barrier function of endothelial cells, which also contributes to drug resistance in GBM [41]. Anti-FGF treatment has been found to have anti-proliferative and anti-angiogenic effects in glioma cell lines [42,43]. GBM is one of the most highly vascularized cancers [44], and FGF-2 acts as an important contributor in the process of angiogenesis [45]. The growth factor promotes angiogenesis directly by activating proliferation and migration of endothelial cells and indirectly by upregulating urokinase-type plasminogen activator, which also leads to cell migration [46]. A recent study reports that survivin, a protein that promotes angiogenesis could trigger the release of FGF-2, along with VEGF, in gliomas and thereby stimulate an increase in growth and proliferation in the tumors [47]. For further discussion of FGFs and FGFRs, as well as a summary of currently existing anti-FGF therapies for cancer, we refer the readers to the comprehensive review by Turner and Grose [48].

Specifically for GSCs, FGF-2 helps to maintain their stem cell state. Its removal from glioma stem cell lines has been shown to result in differentiation, which was not seen when the cells were in the presence of the growth factor [49]. It was recently found that FGF-2 is effective at inducing Nestin, a protein marker for neural stem cells, in C6 glioma cells. This again suggests that FGF-2 contributes to the stemness of glioma cells [50]. Autocrine production of FGF-2 in combination with EGF may also be responsible for retaining the self-renewal potential of GSCs [51].

On the other hand, FGF-2 has been shown to maintain the presence of SP cells in the C6 glioma cell line but was unable to stimulate proliferation without the additional presence of PDGF [52]. Furthermore, GSCs that specifically bear molecular similarities to highly proliferative cell lines were able to rapidly grow in the absence of FGF-2 [53]. These findings suggest that the role of FGF-2 is complex or at least that the murine GSCs may have mechanisms redundant to FGF-2 signaling to maintain their proliferation. Unfortunately, current lack of studies make it premature to comment whether similar ambiguity exists with FGF-2 and human GSCs. The complete role FGF-2 plays in GSCs is thus still largely uncharacterized, and further studies need to be done to uncover its exact effects.

4. Usage of basic fibroblast growth factor in cancer stem cell culture

The use of FGF-2 for *in vitro* culture of GSCs was first established by noting the similarity in culture conditions with neural stem cells [11,13,54]. Lee et al. [26] provided phenotypic and genetic

evidence for the use of FGF-2 (along with EGF) to properly maintain GSC characteristics *in vitro*. More recently Lathia et al. [55] also demonstrated that FGF-2 is critical in maintaining symmetrical propagation of CD133 + GSCs *in vitro*, and that its removal leads to at least twice as many non-stem heterogeneous progeny than at basal levels. Furthermore, of the two exogeneous growth factors used in culture, FGF-2 had a more significant impact than EGF in promoting growth *in vitro* [56]. It is interesting to also note that FGF-2 was found to be important in mediating DNA repair in other cancer cells and CSCs, such as the HeLa cells [57], epidermal stem cells [58,59], and epidermoid carcinoma stem cells [40]. The SP of A549 and H460 non-small cell lung cancer cells also were found to have elevated levels of autocrine FGF-2 [60].

However, a few studies questioned the necessity of adding FGF-2 as a supplement to the GSC *in vitro* media. Li et al. [51] found that primary GBM cells cultured in medium without growth factors still possessed a population of GSCs. Furthermore, they showed that GBM cells expressed FGF-2, suggesting that secretion and autocrine expression of FGF-2 by GSCs may be enough to support growth without the added media supplements. Kelly et al. [61] also found that primary GBM cells can form neurospheres in culture without adding FGF-2 and EGF to the media; however, addition of the growth factors coincided with an increase in sphere proliferation and survival. A more recent study demonstrated that GBM sphere formation increased in the absence of the growth factors and decreased in their presence [62]. It is worth noting, however, that phenotypic alterations in the GBM cells were observed when the exogenous growth factors were added to the media [61]. Maintaining GSCs *in vitro* that are similar to the original tumor cells is critically important, so avoiding any unnecessary alterations should be a top priority when developing culture methods. Therefore, further investigation is needed to determine if the benefits of using FGF-2 in GSC culture outweigh its disadvantages.

5. Comparison of current synthetic analogs of basic fibroblast growth factor

Several different peptides that exhibit similar, but improved biological effects as FGF-2 have been developed, and use of these synthetic analogs rather than FGF-2 in cell culture media could possibly lead to enhanced and more proliferative growth conditions (Table 1). One of the first effective analogs was designed by Ballinger et al. [63]. They designed a peptide named C19jun, which is the combination of a unique 26-residue peptide that can bind FGFR (C19) and a protein domain that can both bind heparin and promote dimerization. Utilizing the same C19 sequence, Pena et al. [64] designed a peptide, F2A3, which combines C19 with an 18 alkyl carbon hydrophobic region connected to a heparin binding domain. An additional analog, F2A4, which is identical to F2A3 except the C19 region is replaced by amino acids 115-129 of FGF-2, was also designed by the same group. When used as a supplement in media, usage of both F2A3 and F2A4 corresponded with higher rates of proliferation than FGF-2, with F2A4 being the highest [64,65]. Another analog, FGF-P, designed by Okunieff et al. has also been shown to improve stem cell survival and proliferation. This 17 amino acid peptide consists of an FGFR binding domain as well as a linking element to allow for receptor dimerization [66].

FGF-2 in media plus the heparin supplement needed to stabilize the growth factor [67] accounts for a large percentage (~30% to 40%) of the cost associated with culturing GSCs *in vitro* (Table 2). Therefore, developing cost-reducing methods for cell culture is another area that synthetic analogs could theoretically show improvement over recombinant FGF-2. It has been proposed that modifying cysteine residues in FGF proteins could decrease the occurrence of incorrect disulfide bridge formation, therefore eliminating the need for reducing agents for FGF stabilization [68,69]. This could not only remove the need for a heparin supplement but also increase biological activity, as less of the growth factor

Table 1
FGF-2 and its patented synthetic analogs.

Peptide	Inventor	Amino acid sequence	Refs.
bFGF	–	MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGV	[70]
bFGF-C78/96S	Fiddes et al.	**C**ANRYLAMKEDGRLLASK**C**VTDECFFFERLGSNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKGV **S**ANRYLAMKEDGRLLASK**S**VTDECFFFERLGSNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS	[69]
C19jun	Ballinger et al.	**AESGDDYCVLVFTDSAWTKICDWSHFRN**GPGGGSGGGSGGGSGGGSGGGSRCGGRIARLEEKVKTLKAQNSELAST ANMLREQVAQLKQKVMNHGGCGGSGGHHHHHH	[63,71]
F2A3	Pena et al.	K – Hex-Hex-Hex-RKRKLERIAR | **NRFHSWDCIKTWASDTFVLVCYDDGSEA**	[64]
F2A4	Pena et al.	K – Hex-Hex-Hex-RKRKLERIAR | YRSRKYSSWYVALKR	[64]
FGF-P	Okunieff et al.	CYRSRKYSSWYVALKRC	[66]

Hex = aminohexanoic acid. Underlined = identical sequence to natural FGF-2. Bold = identical sequence between C19jun and F2A3. Bold+underlined = substitution of Cys with Ser.

Table 2
Approximate cost of GSC media and its components.

Media component	Supplier	Cost	Amount used in media	Total cost	References
bFGF	Invitrogen	$10/μg	10–20 ng/mL	$100-$200/L	[72,73]
EGF	Invitrogen	$1/μg	10–20 ng/mL	$10-$20/L	[72,73]
L-glutamine	Invitrogen	$0.20/mL	2.5 mL/L	$0.50/L	[74]
N-2	Invitrogen	$15/mL	5 mL/L	$75/L	[74]
B-27	Invitrogen	$10/mL	10 mL/L	$100/L	[72]
Heparin	Fisher Scientific	$0.01/μg	5 μg/mL	$50/L	[74]
Neurobasal-A medium	Invitrogen	$0.12/mL	1 L	$120/L	[72]
Total				~$500-$600/L	

is sequestered by heparin, leaving a greater amount for use by cells [69]. Fiddes et al. demonstrated this with their analog bFGF-C78/96S (cysteine residues at amino acids 78 and 96 are replaced by serine residues) that possesses the ability to stimulate higher rates of proliferation than recombinant FGF-2 [69].

Many of these analogs are easier to recover in large, pure quantities due to their small size and exhibit greater stability due to alterations in their amino acid sequences as compared to recombinant FGF-2, which could lead to a longer shelf life [69]. These analogs are still in development phase and are not yet commercially available, and thus their cost figures are not accessible. However, the enhanced stability of some peptides and possible requirement for a smaller media supplement due to increased biological activity suggests that synthetic analogs may potentially be able to eliminate some of the cost associated with FGF-2 use in culture media. Synthetic analogs could be an interesting alternative for *in vitro* culture, and the option should further be explored.

6. Conclusions

With a median survival rate of less than two years, the outcome of most cases of glioblastoma multiforme is a very bleak [1]. In order to improve the prognosis for GBM patients, new therapeutic targets should be explored when developing future treatment options. Two promising areas for exploration are FGF-2 and GSCs. FGF-2 has come to light as a growth factor that plays a significant role in the progression of GBM, contributing to proliferation, angiogenesis, and survival. The growth factor also contributes to drug resistance in GBM, as does the relentless presence of GSCs. Therapies targeting FGF-2 could potentially be effective at destroying GSCs, as it has been discovered that the growth factor is important in preserving the stemness of GSCs [49]. Before this can be done, however, a clearer understanding of FGF-2's exact role in GSCs is essential. Future research should focus on defining this role.

The use of FGF-2 as a supplement in GSC culture medium has become a widely accepted practice. Most agree that the growth factor is critical in order to retain stemness *in vitro*; however, the growth factor may lead to unwanted phenotypic variations in GSCs [61]. Developing treatments based on cells that are unlike those found in primary tumors may lead to therapies that are ineffective in practice. A close examination of the effects of FGF-2 on phenotype should be a priority in order to confirm its usefulness as a media supplement and prevent avoidable mistakes in new drug development.

Studying the effects of synthetic analogs that mimic FGF-2 is another interesting area for potential development. The possibility of enhanced biological activity induced by analogs as compared to recombinant FGF-2 could improve growth conditions and/or reduce the amount of supplement needed in media. In addition, many of these analogs show improved stability over recombinant FGF-2, which could prolong shelf life or eliminate the need for a heparin supplement in media. The costly nature of recombinant FGF-2 and heparin in part accounts for the limited availability of GSCs for research use, and these factors suggest that analogs may be able to eventually reduce the cost, making GSCs more easily accessible. As with recombinant FGF-2, studying the phenotypic effects of any potential analogs would also be important to ensure no significant alterations are caused by the agonists.

Many opportunities exist in improving our understanding of the role of FGF-2 in GSCs. Exploring each of these areas could prove to be very important in the advancement of GBM treatments and GSC *in vitro* culture.

7. Conflict of Interest

The authors declare no conflicts of interest.

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